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Characterization of Antigen-Specific Antigen Processing by the Resting B cell: a Thesis

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CHARACTERIZATION OF ANTIGEN-SPECIFIC ANTIGEN PROCESSING BY THE RESTING B CELL

A Thesis Presented

By

Edmund Joseph Gosselin

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ABSTRACT

Characterization of Antigen-Specific Antigen Processing by The Resting B Cell

(March 1988)

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An optimal antibody response to a thymus-dependent antigen requires cooperation between the B cell and an antigen-specific helper T cell. Major histocompatibility complex restriction of this interaction implies that the helper T cell recognizes antigen on the B cell surface in the context of MHC molecules, and that the antigen-specific B cell gets help by acting as an antigen presenting cell for the helper T cell. However, a number of studies have shown that normal resting B cells are ineffective as antigen presenting cells, implying that the B cell must leave the resting state before it can interact

specifically with a helper T cell. On the contrary, other studies, including those using rabbit Ig as antigen, and rabbit globulin-specific mouse T cell lines and hybridomas, show that certain T cell lines can be efficiently stimulated by normal resting B cells.

One possibility I considered was that small B cells are unable to process antigens, and that the rabbit Ig-specific T cell lines used above recognize native antigen on the B cell surface. In the majority of cases, experiments with B cell lines and macrophages have shown that antigen presentation requires antigen processing, a sequence of events which includes: internalization of antigen into an acid compartment, denaturation or digestion of antigen into fragments, and the return of processed antigen to the cell surface where it can then be recognized by the T cell in the context of class II molecules of the MHC.

The experiments reported here show that the rabbit Ig-specific T cell lines do require an antigen processing step, and that small resting B cells, like other antigen presenting cells, process antigen before presenting it to T cells. Specifically, I show that an incubation of 2-8 hours is required after the antigen pulse before antigen presentation becomes resistant to fixation or irradiation.

Shortly after the pulse, the antigen enters a pronase resistant compartment. Chloroquine, which raises the pH of endocytic vesicles, inhibits presentation. In addition, a large excess of antibody to native antigen fails to block presentation of antigen after a 2-8 hour incubation. Also, although membrane Ig, the antigen receptor on the B cell, is required for efficient presentation of antigen at low concentrations, antigen is no longer associated with the B cell receptor at the time of presentation to the T cell. Modulation of membrane Ig by anti-Ig blocks presentation before but not after the antigen pulse.

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ABBREVIATIONS

MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
Ia	mouse class II MHC gene product
APC	antigen presenting cell
FACS	fluorescence activated cell sorter
ND	not done
Ag	antigen
Ig	immunoglobulin
Rb	rabbit
h	hours
H chain	Rb Ig heavy chain
L chain	Rb Ig light chain
DNP	dinitrophenol
PLL	poly-L-lysine
GL	L-lysine
Ir genes	immune response genes
CI genes	cell interaction genes
LD genes	lymphocyte defined genes
PBS	phosphate buffered saline
FBS	fetal bovine serum
OVA	ovalbumin

INTRODUCTION

B CELL-T CELL INTERACTION IN B CELL ACTIVATION

The humoral phase of the immune response plays a crucial role in the body's defense against both intra- and extracellular pathogens. The key ingredient in this response is antibody, an antigen-specific protein which, once produced, can serve in numerous capacities to either inactivate or destroy the infecting agent. In order for antibody production to occur, a number of events must take place. These events include antigen dependent activation of B lymphocytes specific for the pathogen and factor dependent proliferation and differentiatiation of these cells into antibody secreting plasma cells (1,2).

The production of antibodies to protein antigens has been extensively studied and is known to involve cooperative interactions between the B lymphocyte and helper T lymphocyte (3). However, the precise mechanism by which this interaction occurs is still not thoroughly understood, and continues to generate questions and controversy. While it has been shown that in some instances the addition of T cell factors and antigen is sufficient to induce B cell differentiation and antibody

secretion (4-6), it is generally thought that in the case of the resting B cell, direct cell to cell contact between the B cell and the T cell is necessary in order for B cell activation to occur (3,7).

Linked Recognition

Initial work suggesting that antibody production involves an interaction between two different cell populations was provided in 1966 by Claman et al. (8) who showed that mixtures of bone marrow cells and thymus derived cells, when injected with antigen into an irradiated syngeneic host, produced a better antibody response than either population alone. This was supported by other work which demonstrated complementation between thymus and bone marrow cells in graft vs. host responses (9). In 1968 Miller and Mitchel went a step further, showing that antibody responses in thymectomized mice could be reconstituted by adding back thymic lymphocytes (10). Using semiallogeneic thymus cells, they were also able to demonstrate that the cells responsible for antibody production originated from the thymectomized host and not the donor thymus cells (11). This was in agreement with work by Davies et al. (12) which showed

that thymus cells were unable to produce antibody, while bone marrow derived cells could. This lead to a proposal by Claman and Chaperon in 1969 to explain the phenomenon now termed "thymus-marrow complementation" (13). Called the "two cell theory of antibody production," this model proposed that the thymus derived cell interacted with the antigen, while the cells derived from bone marrow were the antibody forming cells. It was suggested that this interaction might involve direct cell-cell contact between the thymus cell and the antibody producing marrow cell. In 1970 Raff (14) added further support to the "two cell theory of antibody production" by confirming the existence of two subpopulations of lymphocytes using fluorescein labeled antibodies specific for the theta antigen (now called Thy 1), present on thymus cells, and Ig, present on antibody producing cells (ie: bone marrow cells).

Mitchison (15) was the first to provide a tangible model for this interaction. Using adoptive transfer techniques he showed that B cells primed to a given hapten on one carrier would respond to the same hapten on another carrier, providing a population of spleen cells primed to the second carrier was provided. This "carrier effect" (16) could be eliminated if T cells were depleted from the population of spleen cells primed to the second carrier

(17). These results led to the conclusion that the B cell recognized the hapten and the T cell the carrier, that the hapten-carrier complex formed a bridge between these two cells (Fig. 1a), and that through this bridge the helper T cell could deliver to the B cell the appropriate signals required for B cell activation (15,18,19). The term "linked recognition" was coined to describe this process.

MHC Restriction

Allogeneic responses. Prior to the discovery of B cell-T cell interactions, it had been known for some time that genetics played a significant role in the immune response. Tissue transplanted from one individual to a genetically disimilar individual would result in rapid rejection of the transplanted tissue (20). The cause of rejection appeared to be the presence of a number of serologically defined specificities present on cell membranes. The genetic source of these specificities could be mapped to the H-2 locus which is a complex locus or group of closely linked loci, currently referred to as the major histocompatibility complex (MHC) (Fig. 2).

Figure 1

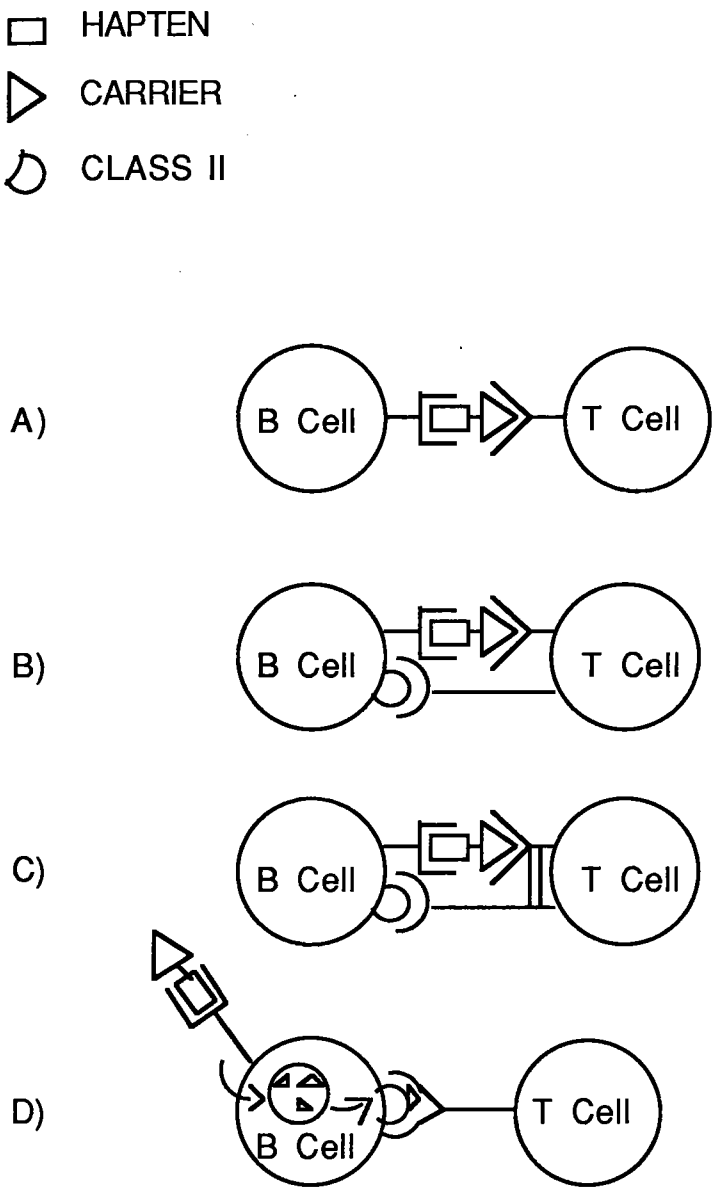
HAPTEN
CARRIER
CLASS II
A)
B Cell
T Cell
B)
B Cell
T Cell
C)
B Cell
T Cell
D)
T Cell
B Cell

Figure 1. Models for B cell-T cell interaction. a) Linked recogniton. The B cell and T cell recognize separate determinants on the same antigen molecule. In the case of a hapten-carrier conjugate, the B cell receptor recognizes the hapten, the T cell receptor the carrier. b) The dual receptor model. The T cell has two separate receptors; one which recognizes the antigen, the other which recognizes Ia. c) The single receptor, dual recognition model. The T cell has a single receptor for both Ia and antigen, but Ia and antigen are recognized separately on the B cell surface. d) Antigen processing. Antigen binds to the B cell receptor, is internalized into an acid compartment where it is processed, and is then returned to the cell surface where a single T cell receptor recognizes antigen and Ia as a molecular complex on the presenting cell surface.

Figure 2

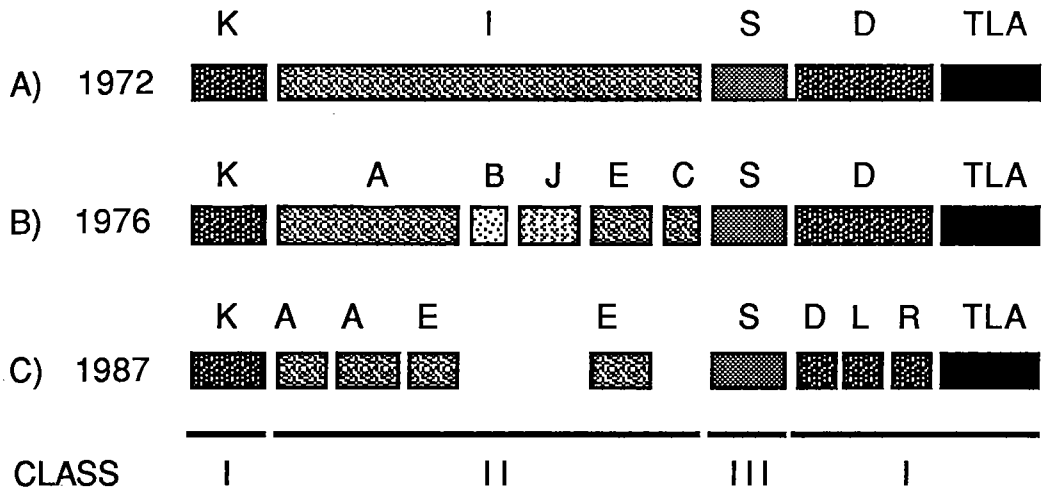
K I S D TLA
A) 1972
K A B J E C S D TLA
B) 1976
K A A E E S D L R TLA
C) 1987
CLASS I II III I

Figure 2. Designations for I region genes in the major histocompatibility region of the mouse. A depiction of how our understanding of the mouse major histocompatibility complex changed from 1972-1987 (21-24).

In 1964 Bain et al. (25) provided some of the first in vitro evidence to indicate that genetics may play a significant role in the regulation of immune cell interactions. When leukocytes from different individuals were mixed, a substantial increase in cell proliferation was observed. This was not the case when cells from monozygotic twins were used. Referred to as the mixed-lymphocyte reaction or MLR, this provided an in vitro system for studying the in vivo phenomenon of H-2 dependent graft rejection. Results by Bain et al. were later supported by studies using one-way mixed lymphocyte cultures in which one of the donor populations was treated with mitomycin C to prevent proliferation (26). In 1966 Dutton (27) provided the most convincing data for a genetic link, showing that in congenic mice a proliferative response could only be obtained providing there were differences at the H-2 locus. Differences at "weaker" histocompatibility loci did not induce a proliferative response. Later studies suggested that it was differences at the K end of the H-2 locus (Fig 2a) which were responsible for the MLR (28). However, these studies did not take into account differences in regions between the H-2K and H-2D loci. It was subsequently demonstrated that the true genetic source of MLR resided

in this, as yet, undefined region between H-2K and H-2D (21,29-31). Because differences in this region could not be easily detected serologically, whereas those in the H-2K and H-2D regions could, the terms LD (lymphocyte defined differences), and SD (serologically defined differences) were applied, respectively, to describe these loci (21).

Antigen-specific responses. During this same period, a number of in vivo studies showed that in order to restore antigen-specific immune function in thymectomized mice it was necessary to provide syngeneic thymus cells (11,32,33). It was suggested that rejection of donor tissue was responsible for the failure of allogeneic cells to reconstitute the antigen-specific response. Kindred and Shreffler, in 1972, provided another explanation (34). They suggested that immune responsiveness was not restored by allogeneic cells due to the positive rather than the negative effect of histocompatibility on cell-cell interactions. They used nude mice which accept allogeneic skin grafts (35-37). In this case, only syngeneic thymus grafts complemented the antibody response, despite the fact that allogeneic grafts were not rejected. An alternative approach to demonstrate the same phenomenon

was used by Katz et al. (38). They injected parental carrier primed T cells and DNP primed B cells into an irradiated F1 hybrid host and measured subsequent antibody responses. Responses were obtained only in those instances where both lymphocyte populations were derived from the same parent (ie: where syngeneic populations were used). Because the strains used in these studies differed in many areas besides the major histocompatibility locus, H-2, it was not possible to definitively conclude what region of the genome was responsible for controlling B cell-T cell interaction. To clarify this issue Katz et al. (39) used congenic strains of mice which differ only in the H-2 locus. These studies demonstrated conclusively that a region in the H-2 locus was responsible for the control of antigen-specific B cell-T cell interactions. More detailed studies using inbred and recombinant strains of mice differing at known locations of the H-2 complex permitted precise location of these genes, referred to as CI or cell-interaction genes (40), again to a region in the H-2 complex between H-2K and H-2D (41).

Immune response genes. Concurrent with the above work, additional studies were being done on a related phenomenon. Using amino acid polymers as antigens,

Benacerraf et al. (42) showed that delayed type hypersensitivity and antibody responses occurred in some, but not all members of an outbred guinea pig population. Use of two different strains of guinea pig definitively showed that responsivenes to these polymers was genetically controlled. Strain 2 guinea pigs were capable of responding to DNP-PLL and to GL whereas strain 13 was not. The F1 hybrid (2 X 13) could also respond suggesting responsiveness was dominant. McDevitt made a similar observation in mice (43) and later mapped the genes controlling immune responsiveness (now termed Ir genes) to an area between the H-2K and S region of the H-2 complex (44).

Unifying theories. Although the common location of the LD, CI and Ir genes suggested a functional as well as genetic link between the three, such a relationship was not accepted in its entirety until the early 1980's. In the mean time several theories were offered to explain this apparent linkage.

In 1972 Benacerraf and McDevitt proposed that Ir genes may code for a T cell receptor, separate and distinct from immunoglobulin (45). A number of studies supported this view (46,47). Efforts to show the presence of

immunoglobulin on the surface of the T cell were largely unsuccessful (46). Yet, by using antibodies derived by reciprocal immunization of congenic mice differing in the H-2K to S region of H-2, directed against Ia antigens (the apparent products of the Ir genes), Ia antigens were localized on the T cell, but not on bone marrow cells (47).

A number of additional studies soon contradicted the latter results, and provided strong evidence against the theory that Ir genes produce a T cell receptor. First it was clearly demonstrated in 1974 that B cells, and not T cells possessed the highest levels of Ia antigens on their surface (48). Functional studies by Katz et al. (49) provided further support for the view that Ir gene control, as well as Ir gene products are expressed by the B cell. Using adoptive transfer, they showed that responses could only be obtained providing the B cell was derived from a responder population. The above data, combined with that of Zinkernagel and Doherty (50) showing that cytotoxic T cells see antigen in the context of class I (H-2K, H-2D), led to the proposal in 1975 and 1976 that Ia antigens may serve a similar function, acting as restriction elements for class II (Ia) mediated antigen presentation (23,51). Subsequent studies strongly

supported this view. Sprent (52) showed that T cells derived from congenic and recombinant strains of mice proliferated in response to antigen only when B cells sharing the K end of the H-2 complex were provided. Similar requirements for other presenting cells were also observed (53). In addition, Ir gene defects on the surface of the macrophage (also an antigen presenting cell) (54-56) or the B cell (49) could alter T cell responsiveness (57-59).

It was primarily the acceptance that LD, CI and Ir genes resided within the same region of the MHC, the I region (57) (Fig. 2a), and the isolation of anti-Ia antibodies (47,60), in particular monoclonal antibodies (61), that allowed researchers to conclusively demonstrate that LD, CI and Ir genes were one in the same, and that their product was the Ia antigen.

In 1976 Schwartz et al. (62) showed that anti-Ia antisera (directed against differences in the I region of the MHC) could block one-way MLR's provided the antibody was directed against the appropriate Ia antigen present on the stimulator cells. Baxevanis et al. (63), in 1980, provided evidence for identity between Ir genes and Ia antigens. First, the presence of certain allelic forms of Ia correlated with immune responsiveness to particular

antigens. Second, responses which localized to different areas within the I region were blocked by antibodies to different allelic forms of Ia. Studies in which it was shown that monoclonal anti-Ia antibody could block antigen-specific responses under Ir gene control, as well as an MLR directed against the same determinant (64), provided the strongest evidence for identity between the three genes (LD, CI, and Ir) and their products. This was confirmed by additional studies using T cell clones in which the responses of antigen-specific and alloreactive clones (responsive to, or restricted by the same allelic form of Ia) could be blocked by the same anti-Ia monoclonal antibody (65). Further evidence for identity between LD, CI, and Ir determinants was provided by studies of an Ia mutant (bm12). Alterations of MLR reactivity, Ir gene function, and restriction of antigen presentation could be attributed to this mutation (66-68).

The Mechanics of MHC Restriction

Early studies by Mitchison and others had suggested that antigen alone served as a bridge to link the B and T lymphocytes (15,16). However, the discovery of MHC restriction forced a serious re-evaluation of this model,

and raised a number of very basic questions, including: what is the gene product regulating restriction, how is this product and antigen recognized simultaneously by the T cell receptor, and how does MHC restriction regulate immune responsiveness.

c. Ia antigens. As discussed above, the answer to the first question, what is the gene product regulating restriction, appeared in a series of studies beginning in 1973 with the isolation of alloantibodies directed against Ia antigens (47,60). By using genetic recombination and anti-Ia antibodies, it was subsequently demonstrated in 1976 (22) that the I region could be divided into five subregions: A, B, J, E, and C (Fig. 2b). Ia antigen determinants were associated with regions I-A, I-E and I-C (69-71). The use of anti-Ia antibodies also facilited biochemical analysis of the Ia protein (22). By using radiolabeling techniques, immunoprecipitation, and PAGE electrophoresis, the product of the I-A subregion was shown to be composed of two chains, alpha and beta, held together by disulfide bonds (72), and weighing approximately 35,000 and 30,000 daltons respectively (71). The I-E/C subregion product was shown to contain an alpha and beta chain (22,70,71,73), however, these chains were

distinct from those produced by the I-A subregion (72,74-76). In addition, multiple amino acid differences were observed between products of the same subregion (77). The observation that the surface expression of the I-E gene product was under the control of two genes (78), one in the I-A and one in the I-E subregion (79) led to the conclusion that I-E beta was actually an I-A subregion product. I-B was defined by phenomenon that could be explained by gene complementation between I-A and I-E (80), no I-C genes or gene products have been isolated (81), and studies in 1984 suggested that the I-J determinant may be the product of genes residing outside the MHC (82). Thus, MHC restriction appears to depend on the surface expression of at least one I region molecule consisting of two polypeptides, alpha and beta, originating from either the I-A or I-A plus I-E subregions of the major histocompatibility complex (Fig. 2c).

The T cell receptor. Prior to characterization of Ia antigens it was already apparent that in order for T cells to be restricted by Ia and also to respond to antigen, they would have to recognize both proteins simultaneously on the same presenting cell. Two major theories were arrived at to explain this dual recognition of Ia and

antigen by the T cell. The first, "The Dual Receptor Model," proposed that the the T cell recognizes antigen and Ia independently (83-90) (Figs. 1b and 1c). The second, "The Single Receptor Model," suggested that the T cell recognizes Ia and antigen as a single molecular complex (90-94) (Fig. 1d). The bulk of the current evidence supports the latter model.

Kappler et al. (95) provided strong evidence against the dual receptor model by showing that when two T cells with different specificities for Ia and antigen were fused (KLH+H-2^f X OVA+H-2^a), hybrids were obtained which recognized parental combinations of Ia and antigen, but did not recognize cross-specificities (ie: OVA+H-2^f or KLH+H-2^a). Attempts to block T cell activation by preincubation of T cells with soluble Ia or antigen were unsuccessful (96). In addition, both the major histocompatibility molecule and antigen must be in the same membrane to induce T cell responses (97-99). Studies have also shown that antigen fragments bind to Ia, binding most avidly to those haplotypes by which their presentation is restricted (100). Furthermore, Ia-antigen conjugates are 10,000 fold more efficient at being presented in artificial membranes than equivalent amounts of Ia and antigen added separately (101). Also, their

presentation can be blocked by the addition of peptides which compete for the same MHC binding site, but not for the same T cell (100). In fact, recent X-ray analysis studies of class I clearly demonstrate that a potential antigen binding pocket does exist between the alpha and beta domains of the class I polypeptide (102).

Other studies which favor the single receptor model include those showing the T cell receptor is composed of a single molecular complex; the Ia and antigen binding site consisting of two polypeptide chains, alpha and beta (103), and studies in which the specificity of a recipient T cell clone was altered to that of a donor cell by transfer of the alpha and beta chain genes of the T cell receptor (104).

Alternatively, it has been suggested that the T cell receptor may still be composed of two separate binding sites for Ia and antigen; the alpha chain binding one ligand, the beta chain the other. A recent study by Saito and Germain (105) showing that transfecting a beta chain gene into a cell can alter restriction could be interpreted as support for this model.

Determinant selection versus clonal deletion Theories to explain the role of MHC restriction in

nonresponsiveness initially appeared in press in 1978. The first, Determinant Selection, was proposed by Rosenthal (56). This model suggested that the inability of certain antigens to elicit an immune response in certain strains is due to the failure of that antigen to properly associate with MHC on the antigen presenting cell. The second model, Clonal Deletion, was proposed by Schwartz, and suggested that nonresponsiveness to certain antigens was due to tolerance induction, the deletion of T cell clones specific for self antigen plus Ia (106). Studies by Ishii et al. (107,108) supported the latter hypothesis by showing that APC from nonresponder animals can present antigen to T cells from responder populations. Klein, in a review in 1984, provided an extensive list of arguements and counter-arguements for both theories (109). Although both may play a role in nonresponsiveness, the most compelling evidence has been provided recently, and is in favor of determinant selection. It has been demonstrated that T cell recognition, and nonresponsiveness do correlate with the ability of certain peptides to bind to the appropriate MHC molecule (100,110,111).

Antigen Presenting Cells

The macrophage. In 1965 Harris (112) observed that in response to antigen in rabbit spleen cell cultures, morphological units of macrophages surrounded by lymphocytes formed. He postulated that these units promoted blast cell formation by the lymphocytes. While this suggested a role for the macrophage in antigen presentation, Mosier (113) provided the first functional evidence in 1967, showing that antibody responses to sheep red blood cells required both an adherent (macrophage) and nonadherent population of cells. Subsequent experiments showed that the nonadherent cells could be further subdivided into two populations (114-116). This led Mosier to propose a model in which the macrophage presented antigen to the T cell, which in turn presented the antigen to the B cell.

The importance of the macrophage as an antigen presenting cell was further emphasized in 1973 when it was demonstrated that T cell proliferation occurred only when antigen pulsed macrophages were provided (117,118). This led Rosenstreich and Rosenthal (117) to conclude that the macrophage or monocyte was "the primary antigen-binding

cell for the induction of T lymphocyte proliferation, in vitro."

These studies and others showing that macrophages were restricted in their ability to present antigen by Ir genes and their products, (Ia antigens) (119-121), set the tone into the early 1980's, during which time the major emphasis on antigen presentation focused on the macrophage. This limited view of antigen presentation culminated in 1979 in a study by Singer et al. (122) in which it was suggested that previous studies (123-126) showing MHC restriction between the B cell and the T cell were incorrect owing to contamination of these cell preparations with macrophages. Singer et al. (122) went on to demonstrate that the interaction between antigen presenting cells (ie: the macrophage) and T cells was MHC restricted while the interaction between B and T cells was not.

The B cell. The view that the macrophage was the primary antigen presenting cell in the immune response changed rapidly following the appearance of a number of studies in 1981 and 1982 in which it was shown that the B cell could also serve as an antigen presenting cell (18,127-129). Chesnut and Grey (127), in 1981, showed

that normal B cells could present rabbit anti-Ig to rabbit globulin-specific T cells. Jones and Janeway (18), also in 1981, demonstrated that the B cell could present antigen, and showed that the presentation was MHC restricted. They also suggested that the purpose of the antigen presenting macrophage might be to activate T cells prior to a T cell-B cell interaction. In 1982, Glimcher et al. (129) published work in which it was demonstrated that a number of B cell lymphomas could present antigen to their antigen-specific T cells. Walker et al. (128) confirmed these observations, while also showing that the majority of monocytic and macrophage tumors tested could not present because they lacked Ia. A large number of subsequent studies confirmed the above observations that B cells present antigen (1,130,131).

Initial attempts to demonstrate antigen presentation by normal inactivated B cells were not successful (132-134) owing to the sensitivity of these cells to irradiation (135). T cell proliferation was often used as an indicator of presentation and irradiated B cells were routinely used when measuring T cell proliferation. In 1984 Ashwell et al. (135) showed that when radiation sensitivity was taken into account, normal B cells, both small and large, could present a number of antigens

including GAT, Cytochrome c and hen egg lysozyme, with equal efficiency, to T cell lines and T cell hybridomas. Frohman and Cowing (136), in 1985, showed that lymph node primed B cells could also present antigen (KLH) to primed T cells. However, upon comparing the efficiency of presentation between macrophages, LPS activated B cells and small resting B cells, they observed that the latter two were 4 and 1000 fold less efficient, respectively, at presenting KLH. In contrast to this observation, Tony et al. (137,138) showed that resting B cells could present nanogram levels of rabbit anti-Ig to rabbit Ig-specific T cell lines.

Other antigen presenting cells. Recent studies have suggested that antigen presentation is not limited to the B cell and the macrophage. Other antigen presenting cells include: the dendritic cell (139,140), the endothelial cell (141-143), and most probably a number of additional Ia bearing cells (131). The primary requirement for antigen presentation appears to be the presence of Ia on the presenting cell surface. This is most clearly demonstrated in studies in which Ia and antigen are placed in a lipid bilayer, and presentation is observed (144,145). For most protein antigens, there appears to be an additional requirement, antigen processing.

Antigen Processing

As originally understood, linked recognition implied that the hapten-carrier complex remained unaltered following its initial binding to the B cell receptor, and that both the T cell and the B cell recognized the native antigen. Within the last ten years numerous studies have suggested that this interpretation of linked recognition is incorrect (131,146-149). First, a revised model (Figs. 1b and 1c) was necessary to account for the fact that such interactions are restricted only to those presenting cells carrying the appropriate class II molecule on their surface (94,146). Second, it has been shown that most B cells primed to native antigen see only native antigen, while T cells, primed in a similar manner, respond to both native and denatured antigen (150,151). Third, studies using macrophages as antigen presenting cells (APC) have indicated that in order for the T cell to interact with Ia and antigen on the macrophage surface, the antigen must be processed (ie: internalized into an acid compartment, structurally altered, and returned to the cell surface in the context of class II where it can then be recognized by the T cell) (152,153). In addition, numerous studies

using B cell lymphomas (154,155) and antigen-specific EBV transformed B cells (147) as APC have indicated that B cells must also process antigen before presenting it to the T lymphocyte. This has led to the proposal of an alternative model to explain the phenomenon of linked recognition, which includes antigen processing (Fig. 1d). In this model the recognition of antigen is sequential rather than simultaneous and involves both native and processed forms of the antigen. First the B cell receptor recognizes the native antigen in solution, then the antigen is internalized, and is processed. The processed antigen is then returned to the B cell surface were it is recognized by the T cell in the context of MHC class II molecules.

Despite the overwhelming data in favor of processing, a number of researchers continue to provide evidence which supports presentation of native antigen. Klein and Walden (156) showed that native antigens such as insulin and ovalbumin could be presented when inserted into vesicles containing class II. However, it has been argued that the insertion of antigen into these vesicles denatures them, providing the equivalent of processing.

Ziegler (157) showed that large molecular weight bacterial antigens can be presented by fixed presenting

cells, and that in the absence of fixation, chloroquine, which blocks presentation of many processed antigens, does not block presentation of these antigens.

The most convincing evidence for presentation of native antigen however, has been provided by Allen's laboratory. He has shown that fibrinogen is presented to T cells in its native form (158) on fixed presenting cells, and that this presentation is not chloroquine sensitive. This work enjoys considerable acceptance even by those like Allen, who support the antigen processing model. This is primarily because fibrinogen contains minimal secondary structure in the region recognized by the T cell, and therefore may closely resemble denatured, or processed antigen in that region.

ANTIGEN-SPECIFIC ANTIGEN PRESENTATION

Experimental Models

An optimal antibody response to a thymus-dependent antigen requires cooperation between the B cell and the helper T lymphocyte specific for the same antigen molecule. MHC restriction of this interaction implies

that the helper T cell recognizes antigen on the B cell surface in the context of the class II MHC molecules, and that the antigen-specific B cell gets help by acting as an antigen presenting cell for the helper T cell.

Due to the clonal distribution of B cell specificities, antigen-specific B cells are rare, and presentation by these cells is difficult to study. A number of methods have been used to circumvent this problem. These include the use of antigen-specific EBV transformed B cell lines (147), purified antigen binding cells (159), and rabbit anti-mouse Ig in place of antigen (137,138). The latter model, anti-Ig, provides a polyclonal antigen analogue which binds to all Ig+ B cells regardless of their specificity. Initially, it provided a model for studying B cell activation as a direct consequence of antigen binding (160). Chesnut and Grey (127) extended its use to the study of T cell activation by demonstrating that rabbit Ig-specific T cells would respond to rabbit anti-mouse Ig initially bound to mouse B cells. The isolation of rabbit Ig-specific T cell lines by Tony and Parker in 1985 (138) has now made it possible, with this model (Fig. 3), to study the full range of events involved in T cell dependent B cell activation.

Figure 3

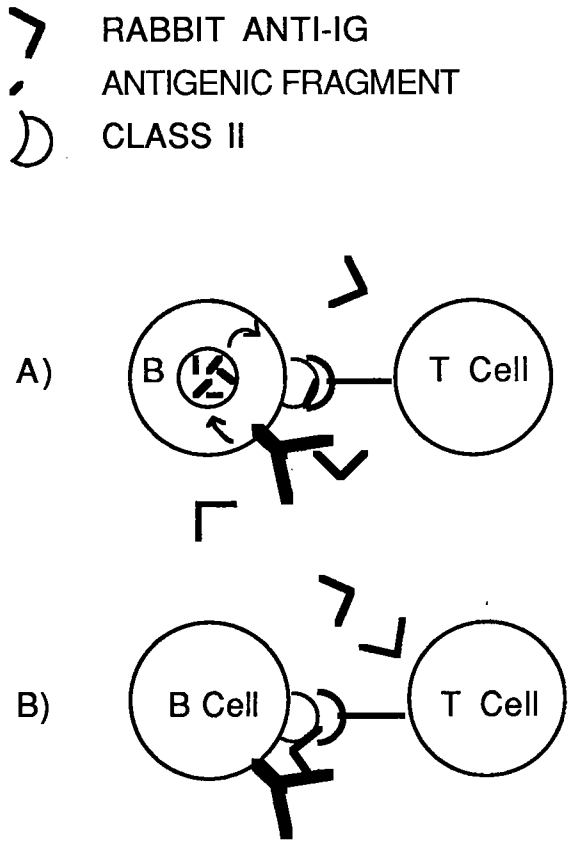
RABBIT ANTI-IG
ANTIGENIC FRAGMENT
CLASS II
A)
B
T Cell
B)
B Cell
T Cell

Figure 3. Models for the presentation of rabbit anti-mouse Ig by resting B cells. (a) Antigen processing. Rabbit anti-mouse Ig binds to Ig on the surface of mouse B cells. The rabbit Ig is then internalized into an acid compartment, processed, and returned to the cell surface where it is presented in the context of class II. (b) Presentation of unprocessed antigen. Rabbit anti-mouse Ig binds to the mouse B cell and, while still bound to surface Ig, is presented to the T cell in the context of class II.

Although a number of studies have been done to demonstrate that B cells are very efficient antigen presenting cells for antigen initially bound to membrane Ig (127,137,138,147,161,162), it is still unclear at what stage in the activation pathway the B cell can present antigen to the T cell in order to obtain T cell help.

Normal B Cells

Normal B cells are heterogeneous in size, RNA content, surface molecule expression, and their ability to function in antibody responses. These differences have been interpreted as representing different states of activation within the normal B cell population (7,160). Despite the difficulties of studying antigen presentation by normal antigen-specific B cells, receptor-mediated antigen presentation by the normal B cell has been demonstrated in a number of experimental systems, including those using macrophage depleted spleen cells (127), primed lymph node B cells (161), and antigen-specific B cells isolated from normal B cell populations (162). In the latter system antigen presentation has been shown to involve antigen processing such that the antigen, although initially bound

to membrane Ig, is no longer associated with the B cell receptor at the time of presentation. Also, antigen presentation by these cells to a helper T cell line results directly in vigorous antibody production by the presenting B cells (163).

Since all of the above studies used normal B cell populations, these studies also included activated B cells. Therefore, it is not possible to conclude at what stage in B cell activation B cells are able to present antigen.

Resting B Cells

The majority of small, resting B cells differ from B cells in various states of activation (ie: normal B cell populations), in their requirements for T cell help, and in their ability to act as antigen presenting cells (1,7,130,131). Even when sensitivity to irradiation has been taken into account, large differences in the ability of resting versus activated B cells to act as antigen presenting cells have been reported in some (136,164,165), but not all laboratories (135,137,138). Thus, the question as to whether resting B cells do or do not process and present antigen in a fashion similar to that of other presenting cells remains controversial.

Chestnut and Grey suggested in 1981 that normal B cells could present rabbit anti-Ig to rabbit Ig-specific T cells, but could not present normal rabbit globulin (127). This conclusion was later retracted, and it was suggested that contamination with large B cells, and/or activation with anti-Ig had been responsible for the presentation of rabbit anti-Ig which was observed (132).

As previously discussed, in 1984 Ashwell et al. (135), using antigens that bind nonspecifically to the B cell surface, provided evidence not only that resting B cells present antigen, but that they do so as efficiently as large cells. In addition, they concluded that the previous inability of Chesnut and Grey to demonstrate presentation by these cells was owing to the fact that they had irradiated their presenting cells prior to the addition of antigen.

Since Ashwell's paper in 1984, additional studies, using both receptor Ig-specific and nonspecific antigens, have been published to indicate that resting B cells do not present antigen (131,164,165), or are very inefficient at doing so (136). In direct contrast to the latter studies, our laboratory has demonstrated that resting B cells do present rabbit globulins, both normal and anti-mouse Ig specific, to our rabbit globulin-specific T cell

lines (138). It therefore seems clear that at least in this case resting B cells do present antigen. The question that remains is how?

One possible explanation I considered was that resting B cells are unable to process antigen (Fig. 3a), and that our T cell lines, which were isolated based on their ability to induce B cell activation and differentiation in the presence of rabbit globulin and resting B cells, may well have been selected to see native rabbit Ig (Fig. 3b).

OBJECTIVE

The purpose of this study was to determine if resting B cells are able to process antigen. Although in most studies, T cell clones see only processed antigen, it is not clear that processing is a prerequisite for presentation of all antigens to all T cells (157,158,166). In addition, processing of receptor-bound antigen had not been thoroughly characterized in normal B cells which could be clearly defined as resting.

The use of size-selected small B cells and the anti-Ig model circumvents the problem of using B cell preparations contaminated with activated B cells. This method provides a polyclonal system for studying antigen processing and

presentation by B cells which can be clearly defined as resting.

I report here that small resting B cells are able to process antigen.

MATERIALS AND METHODS

REAGENTS

Rabbit Fab' and $F(ab')_2$ anti-mouse IgM, $F(ab')_2$ anti-mouse IgD, $F(ab')_2$ anti-mouse Fab, and $F(ab')_2$ NRG, which were used as antigens in these studies, were made by myself and technicians in our laboratory, and were obtained as previously described (137,167). Affinity purified $F(ab')_2$ goat anti-mouse Ig and affinity purified $F(ab')_2$ goat anti-rabbit Ig were purchased from Jackson Immunoresearch Laboratories Inc. (Avondale, PA.). The monoclonal rat anti-IgM antibody (b-7-6) (168) was a gift of Lyn Schmidt (U. Mass. Medical Center, Worcester, MA.). Antibodies used in T cell depletion were obtained as previously described (137,169). Heavy and light chains of rabbit Ig were a gift of Dr. Thomas Kindt (NIH). Normal rabbit globulins of known allotype were a gift of Dr. Rose Mage (NIH). The cell line producing M5/114.15.2 (an anti-Ia antibody) was obtained from the American Type Culture Collection (Rockville, MD.), and was isolated as previously described (170,171). Chloroquine was purchased from Sigma.

B CELLS

A20 (172) and L10 are H-2^d B cell lymphomas and were obtained from Laurie Glimcher (Harvard Sch. of Publ. Hlth.). Unless otherwise indicated, small B cells were obtained from the spleens of C3D2(C3H/HeJ X DBA/2J)F1, (H-2^k X H-2^d) mice (Jackson Laboratory, Bar Harbor, ME.). In some instances CBA/N X A.BY (H-2^k X H-2^b) mice (Jackson Laboratory, Bar Harbor, ME.) were used. Spleen cells were depleted of T cells using a cocktail of monoclonal antibodies (137,169): mouse anti-Thy 1.2 (HO-13-4), rat anti-Thy 1 (J1j.10), rat anti-Lyt-2 (3.168.8), rat anti-L3T4 (GK 1.5.6), and mouse anti-rat kappa (MAR 18.5). Subsequent to the addition of the above antibodies, agarose absorbed guinea pig complement (Pel-Freez, Brown Deer, WI.) was added. Small, resting B cells were separated from activated B cells and other kinds of spleen cells by counterflow centrifugal elutriation (138). Size profiles of these cell populations were determined with a Coulter Counter and Channelyzer (Coulter Electronics, Woburn, MA.)(Fig. 4).

Figure 4

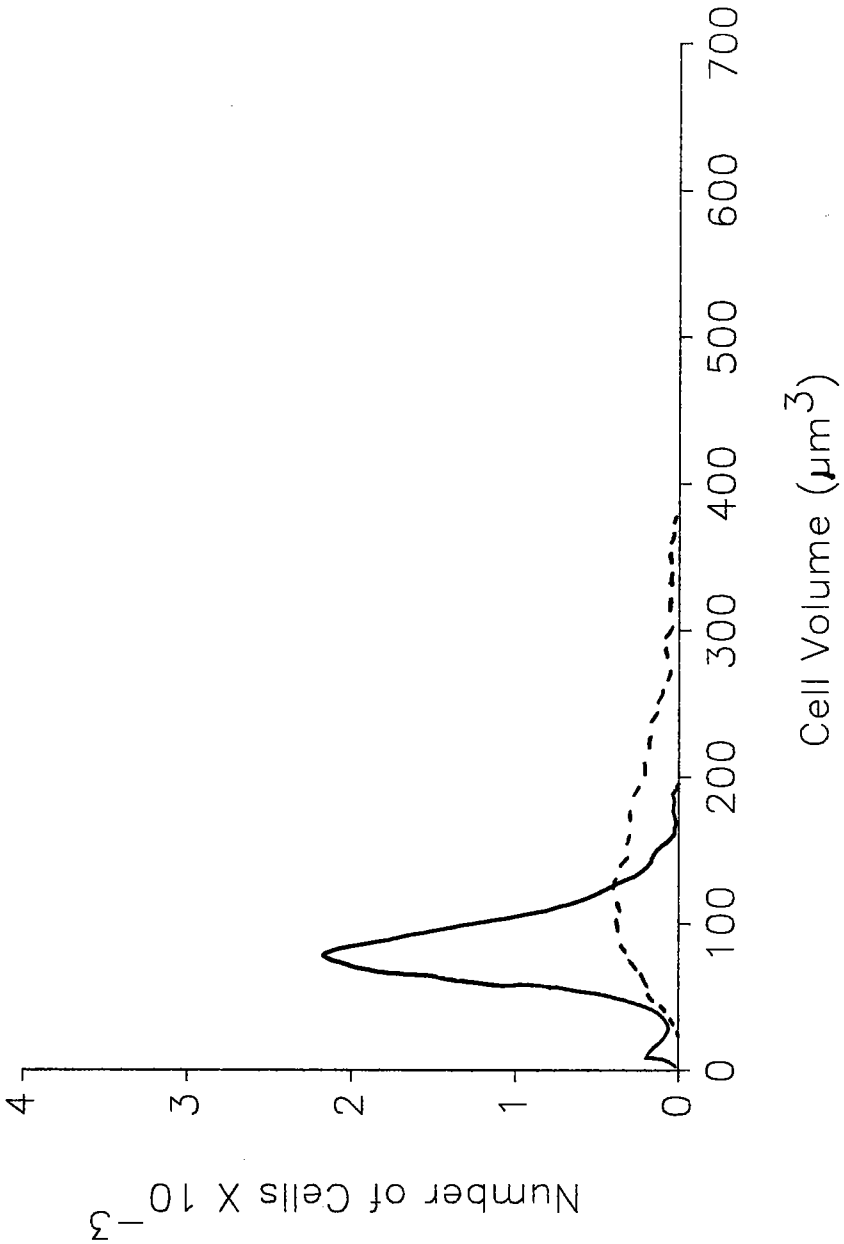
Number of Cells X 10^-3
0
1
2
3
4
0
100
200
300
400
500
600
700
Cell Volume (μm^3)

Figure 4. Size profiles of B cells isolated by centrifugal elutriation. The first peak (————————) represents small B cells, the second peak (-------) the small and large cells which remain after collection of the intial small cell fraction. B cells were obtained from spleen cells depleted of T cells prior to elutriation.

T CELLS

DO 54.8 was a gift of Howard Grey and is an H-2^d restricted, OVA-specific T cell hybridoma (154). D10 was obtained from the American Type Culture Collection (Rockville, MD.) and is a conalbumin-specific, H-2^k restricted helper T cell line. D10 is alloreactive to H-2^b. Two rabbit globulin-specific T cell lines were used in these studies, CDC25 and CDC35. These T cell lines were isolated by David Parker in our laboratory and were maintained by myself and technicians. CDC25 is H-$2^{k/d}$ restricted (it responds to antigen on F1 antigen presenting cells but not on APC of either parent) and CDC35 is H-2^d restricted. The isolation and maintainance of these lines is described elsewhere (137,138). In brief, antigen-specific T cells were established from lymph node cells of C3D2 mice primed in vivo with $F(ab')_2$ NRG and boosted in vitro with 100 µg/ml $F(ab')_2$ NRG. Lines were established by limiting dilution at 100 input cells/well. T cells were tested for their ability to support $F(ab')_2$ anti-Ig dependent B cell proliferation and Ig secretion. Active cells were maintained by restimulation of 6 X 10^4 T cells with 2 X 10^6 irradiated

spleen cells per ml of medium, plus 100 µg/ml of $F(ab')_2$ NRG and IL-2 from rat spleen Con A supernatant. Although it was initially reported that these cell lines secrete IL-2, we have since found that these T cell lines make IL-4, and no detectable IL-2 (173, and our unpublished results).

CELL CULTURES

I set up cultures containing B and T lymphocytes in 96 well, half area, flat-bottomed plates (Costar no. 3696). Each well contained 5 X 10^4 B cells and 1 X 10^4 T cells in 100 µl of RPMI 1640 (GIBCO Laboratories, Grand Island, NY), containing 50 µM 2-mercaptoethanol, L-glutamine, penicillin, streptomycin, gentamycin and 10% FBS. B cells were either pretreated with antigen or antigen was added directly to each well. Subsequently, cultures were incubated at 37°C for 24 hours. Supernatants were then collected and assayed for lymphokine content as a measure of the T cell activation resulting from presentation of antigen.

LYMPHOKINE ASSAY

To each full area well (Costar no. 3598) containing 50 µl of supernatant, I added 100 µl of RPMI 1640 containing 1 X 10^4 IL-2/IL-4 dependent T cells (CTLL-2). Cultures were then incubated at 37°C for 24 hours. ^3H thymidine (1 uCi/well, 2 Ci/mmole, New England Nuclear, Boston, MA.) was added for an additional six hours, at which time the cells were harvested onto glass fiber filters and washed with water before liquid scintillation counting.

FIXATION

In order to fix cells prior to presentation I used a modification of procedures by Shimonkevitz et al. (154). Cells were washed twice with HBSS (no FBS) and resuspended in the same to a final concentration of 5 X 10^6 cells/ml. An equal volume of 0.1% glutaraldehyde (Grade I, Sigma) was then added for 30 seconds, followed by an equal volume of 0.2M L-lysine (Sigma). Cells were then washed with HBSS (no FBS) and HBSS (1.0% FBS), and resuspended in RPMI 1640, with additions, to a final concentration of 1 X 10^6 cells/ml.

PRONASE TREATMENT

To remove antigen with pronase I again used a modification of methods originally used by Chesnut et. al. (155). Three milliliter samples of cells at 1 X 10^6 cells/ml were washed once with RPMI 1640 (no FBS) and resuspended in 0.5 ml of the same to give a final concentration of 6 X 10^6 cells/ml. To each sample of cells, 50 µl of pronase (Type XIV, Sigma) dissolved in double distilled water, and 5 µl of DNase (Grade II, Boehringer Mannheim GmbH) dissolved in HBSS (1.0% FBS) were added to give final concentrations of 1.0 mg/ml and 10 µg/ml respectively. Cells were then incubated for 30 minutes at 37°C and the reactions stopped by the addition of 0.5 ml of 100% FBS. Cells were then washed once in HBSS (1.0% FBS) and resuspended in RPMI 1640 to a concentration of 1 X 10^6 cells/ml.

FACS ANALYSIS

Fluorescence staining was carried out as previously described (137). B cells were adjusted to 1 X 10^6/ml in

PBS containing 1.0% FBS and 0.1% sodium azide. Cells were then incubated for 30 minutes at 4°C with the appropriate fluorescein conjugated antibody at a saturating concentration. The cells were then washed and fixed. Flow cytometry was performed by technicians in the flow cytometry facility (U. Mass. Medical Center) on a fluorescence activated cell sorter (FACS) 440 (Becton-Dickinson Immunocytometry Systems, Mountain View, CA.).

RESULTS

A PROCESSING INTERVAL IS REQUIRED PRIOR TO PRESENTATION OF RABBIT IG BY THE RESTING B CELL.

Previous studies with presenting cells other than the resting B cell have shown that antigen presentation can be blocked if the presenting cells are fixed before or immediately after addition of antigen. The same cells will present if fixed following a 37°C incubation after antigen addition (155). Irradiation has been shown to have a similar effect on normal B lymphocytes (161). Using these same methods I wanted to determine if there is a processing time required before resting B cells can present rabbit Ig to our T cell lines. Fixation studies in which B cell lymphoma cells were incubated for varying amounts of time with antigen, at 37°C, followed by removal of the antigen and immediate fixation, suggested that a processing time for rabbit Ig was required by CDC35 prior to presentation (Table 1). It also showed that the processing time for rabbit Ig was similar to that of OVA, between 4 and 20 hours (Table 1). Similarly fixation

TABLE 1

Processing Kinetics of Rabbit Ig by B cell Lymphomas[a]

B Cell	A20				L10			
	Antigen							
Time (h)	None	anti-IgM	NRG	OVA	None	anti-IgM	NRG	OVA
0.5	250	285	315	283	329	317	338	225
2.0	466	258	299	329	261	372	42	325
4.0	394	3757	2519	1026	221	1698	799	1137
20.0	509	8558	13198	20863	255	5704	8464	10388

[a] B cells were adjusted to 1 X 10^6 cells/ml and incubated with either no antigen, 10 µg/ml $F(ab')_2$ anti-IgM, 1.0 mg/ml $F(ab')_2$ NRG, or 1.0 mg/ml OVA. Cells were incubated for 0.5, 2, 4, or 20 hours at 37°C in HRPMI 1640 at which time antigen was washed away and the cells were fixed. B and T cells, CDC35 or DO 54.8, were then put in culture and lymphokine secretion measured.

and irradiation (2000 rad) of small B cells implied a requirement for processing by both T cell lines (CDC25 and CDC35) (Table 2). If B cells were treated at time 0 (immediately after the 4°C antigen pulse), presentation was blocked, but if they were treated following an 8 hour 37°C incubation with antigen, presentation was observed (Table 2). Fab' and $F(ab')_2$ anti-Ig gave similar results (Table 2). Although irradiation routinely reduced overall responses by 1/3 to 1/2 (Fig. 5), irradiation appeared to be the more sensitive method giving higher responses with equivalent doses of antigen (Table 2). Additional studies using only irradiation, in which antigen was washed away immediately after a 30 minute 4°C pulse in the presence of 0.1% sodium azide, indicated that the minimal time needed to obtain presentation was actually between 2 and 4 hours, and that a variety of antigens including nonspecific $F(ab')_2$ NRG, and a number of receptor-specific rabbit globulins: $F(ab')_2$ anti-Fab, $F(ab')_2$ anti-IgD, $F(ab')_2$ anti-IgM, Fab' anti-IgM, produced similar results (Fig. 5).

These experiments also showed a somewhat longer processing time requirement by CDC25 for rabbit anti-IgM and $F(ab')_2$ NRG, and a consistent reduction in the level

TABLE 2

Use of Fixation Versus Irradiation in Measuring Processing Kinetics of Small B Cells[a]

Treatment	Fixation				Irradiation			
	Antigen (μg/ml)							
Time (h)	(0)	$F(ab')_2$ (0.1)	$F(ab')_2$ (1.0)	Fab' (0.1)	Fab' (1.0)	$F(ab')_2$ (0.1)	$F(ab')_2$ (1.0)	Fab' (0.1)	Fab' (1.0)
CDC25									
0[b]	362	8212	11169	4651	11058				
0	423	725	589	640	455	415	ND	528	1410
8	315	1564	4169	718	2266	7531	9724	7360	7860
20	268	1081	4949	1089	2195	7622	9840	6945	8429
CDC35									
0[b]	638	6550	9820	2610	8253				
0	299	696	276	785	409	571	639	481	383
8	286	766	570	502	407	4745	7034	5946	5659
20	334	584	707	781	402	2890	7370	3671	5226

[a] B cells were pulsed in RPMI 1640 for 30 minutes at 4°C with varying doses of antigen, then incubated at 37°C still in the presence of antigen. At 0, 8 or 20 hours of incubation the antigen was washed away and the cells were resuspended in RPMI 1640 to a final concentration of 1 X 10^6 cells/ml. After the wash cells were divided into three groups. The first group remained untreated, the second group was fixed, and the third group was irradiated with 2000 rad (from a cesium source). Immediately following treatment, T cells were added and lymphokine secretion measured.

[b] Untreated.

Figure 5a

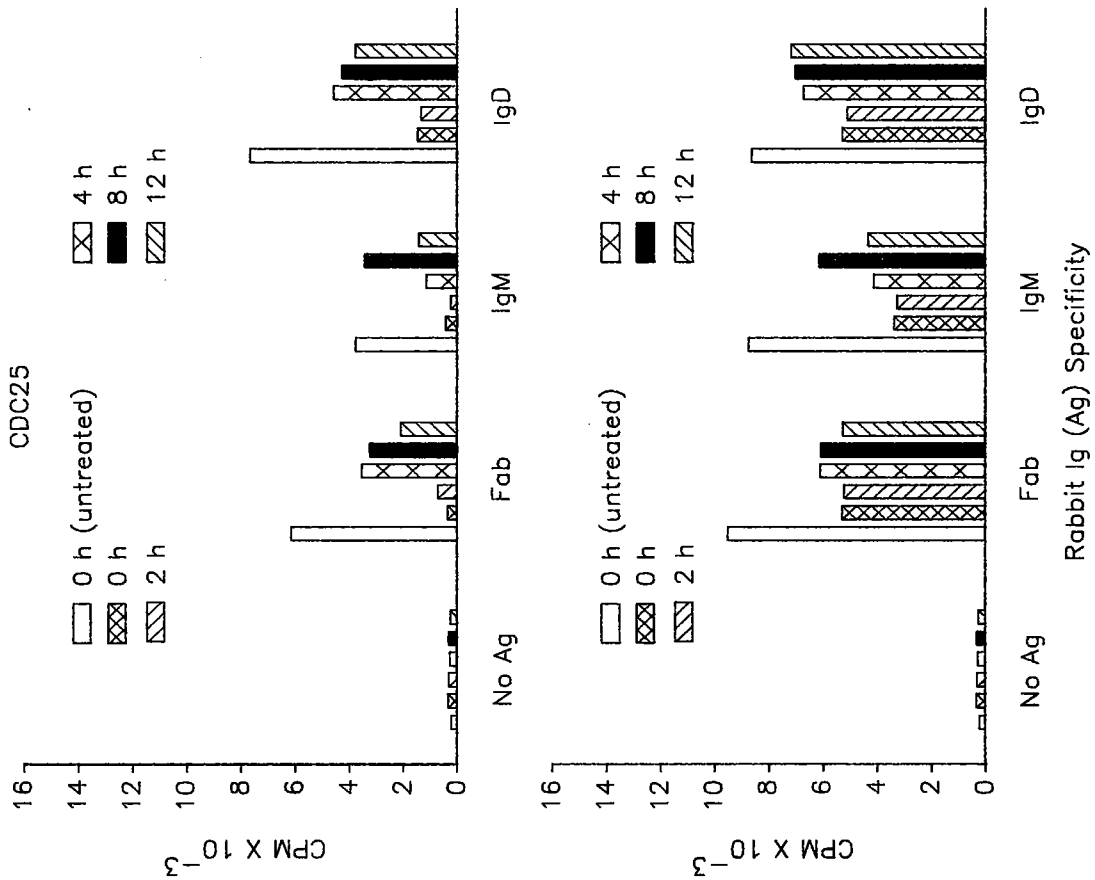
CDC25
0 h (untreated)
0 h
2 h
4 h
8 h
12 h
CPM X 10^-3
16
14
12
10
8
6
4
2
0
No Ag
Fab
IgM
IgD
Rabbit Ig (Ag) Specificity

Figure 5b

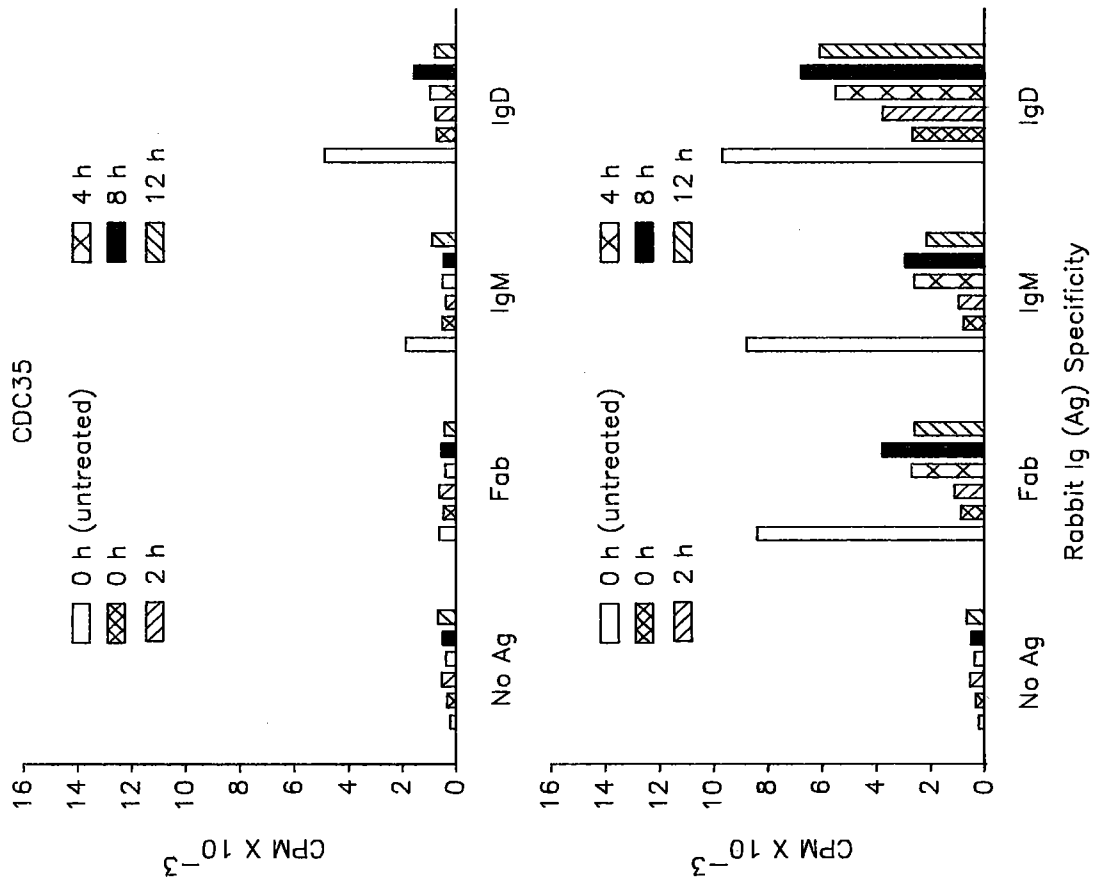
CDC35
0 h (untreated)
0 h
2 h
4 h
8 h
12 h
CPM X 10^-3
No Ag
Fab
IgM
IgD
Rabbit Ig (Ag) Specificity

Figure 5c

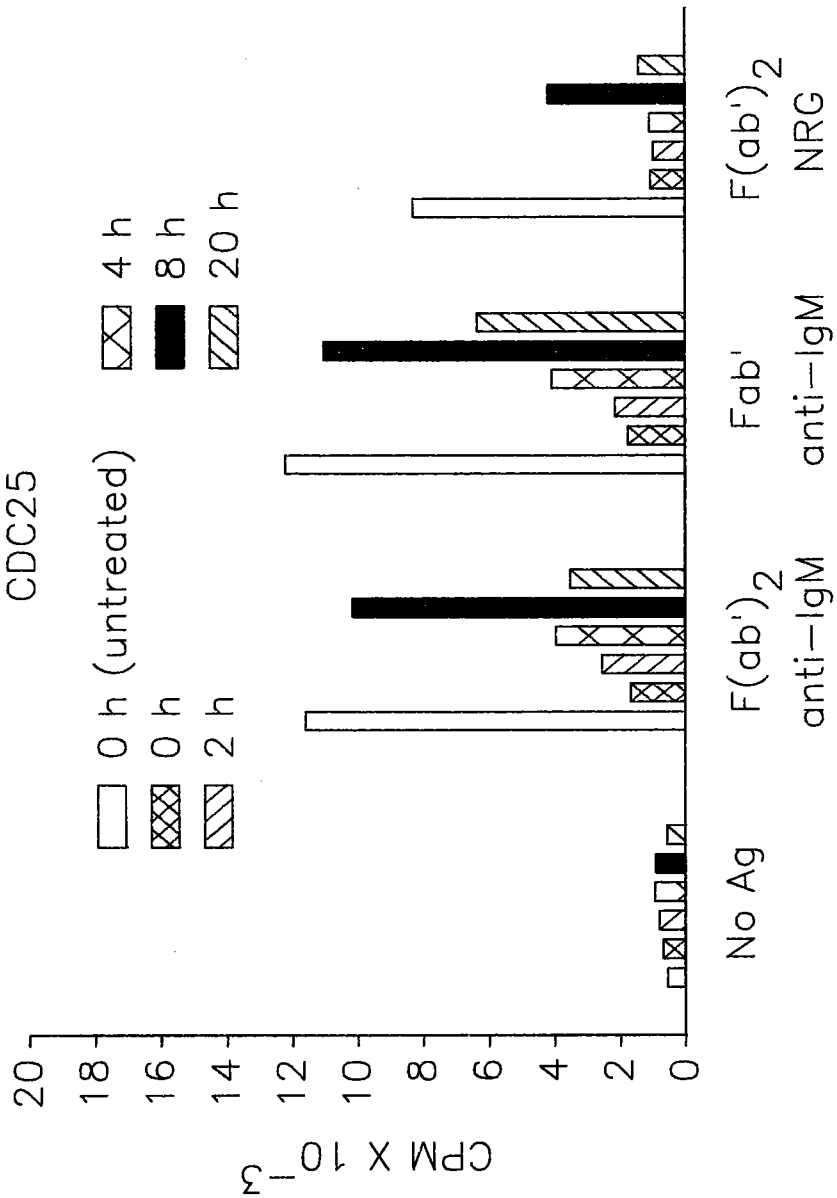
CDC25
0 h (untreated)
0 h
2 h
4 h
8 h
20 h
CPM X 10^-3
0
2
4
6
8
10
12
14
16
18
20
No Ag
F(ab')2 anti-IgM
Fab' anti-IgM
F(ab')2 NRG

Figure 5. Processing kinetics of receptor-specific and nonspecific rabbit Ig. Resting B cells were pulsed in RPMI 1640 (0.1% sodium azide) for 30 minutes at 4°C with $F(ab')_2$ anti-Fab, $F(ab')_2$ anti-IgM or $F(ab')_2$ anti-IgD (a and b), or (c) F(ab')2 anti-IgM, Fab' anti-IgM or $F(ab')_2$ NRG. Cells in figures 5a and 5b were pulsed with 0.1 µg/ml (top figure) or 1.0 µg/ml (bottom figure) of antigen. In figure 5c cells recieved 1.0 µg/ml of antigen except in the case of $F(ab')_2$ NRG which was added at 1.0 mg/ml. Immediately after the pulse, antigen and azide were washed away and the cells incubated in RPMI 1640 at 37°C. At 0, 2, 4, 8, 12 or 20 hours of incubation, cells were irradiated with 2000 rad. Immediately following irradiation T cells, CDC25 (a and c) or CDC35 (b), were added. Lymphokine secretion was measured 24 hours after addition of T cells.

of presentation after incubations of 12 or 20 hours at 37°C. A substantial increase in the level of presentation by resting B cells irradiated at time 0 could be obtained by increasing the dose of antigen (Fig. 5a).

To determine if presentation of antigen by irradiated cells at 4 and 8 hours might be due to the induction of radioresistance by the antigen, resting B cells were preincubated with goat anti-Ig (1.0 µg/ml) for 8 hours at 37°C. This did not alter the susceptibility of resting B cells to irradiation at time 0 (Table 3), indicating that, in the case of receptor-specific antigen, presentation seen at 4 and 8 hours was not due to induction of radioresistance by the antigen.

THE RELEVANT ANTIGEN IS INTERNALIZED.

It is generally believed that processing takes place inside the presenting cell. Thus, native antigen, once bound to the presenting cell, should be susceptible to pronase digestion unless it is internalized. Once inside the cell, antigen should be protected from pronase. In order to determine if the rabbit Ig is inside the resting B cell during processing, cells were pulsed at 4°C,

TABLE 3

The effect of Receptor Crosslinking on the Radiosensitivity of Small B Cells[a]

Antigen (Ag) (µg/ml)	Untreated		Goat Anti-IgM	
	Ag->Irr.	Irr.->Ag	Ag->Irr.	Irr.->Ag
0	336	493	305	220
0.01	9560	355	9837	532
0.1	24372	10423	21887	11106
1.0	25909	19371	18077	19316

a B cells were divided into two groups. One group remained untreated while the other was pulsed with 1.0 µg/ml goat anti-IgM as described in figure 5. At the end of 30 minutes antigen and azide were washed away and the cells were incubated for 8 hours at 37°C. Each group of B cells was further divided in half and one half was irradiated immediately with 2000 rad while the other was not irradiated until the completion of an 8 hour, 37°C incubation with antigen (Rabbit $F(ab')_2$ anti-Fab). B cells were placed in 96 well plates immediately following irradiation, T cells (CDC35) were then added, and, in the case of unpulsed cells, antigen.

the antigen was washed away, and the cells were treated with pronase at varying times during a 37°C incubation to remove surface bound antigen (155). In instances where B cells were pulsed with low doses of antigen, treatment with pronase immediately after the 4°C pulse reduced presentation as much as 80%, with the level of inhibition gradually decreasing to 0% by 2-4 hours (Fig. 6). These data suggest that the majority of relevant, native antigen, is inside the cell and protected from pronase by 2-4 hours.

I also considered another explanation for pronase resistance. It might be that processing itself alters the susceptibility of antigen to pronase. In order to distinguish this possibility from that of internalization, pulsed B cells were treated with pronase at 2, 4 or 8 hours, and were then irradiated at 8 hours to prevent further processing. Diminished responses were observed at 4 and 8 hours. This result suggests that processed antigen on the cell surface can be removed by pronase, and that irradiation prevents additional processed antigen from reaching the cell surface.

The level of inhibition by pronase treatment alone, or by irrradiation and pronase treatment at 8 hours was

Figure 6a

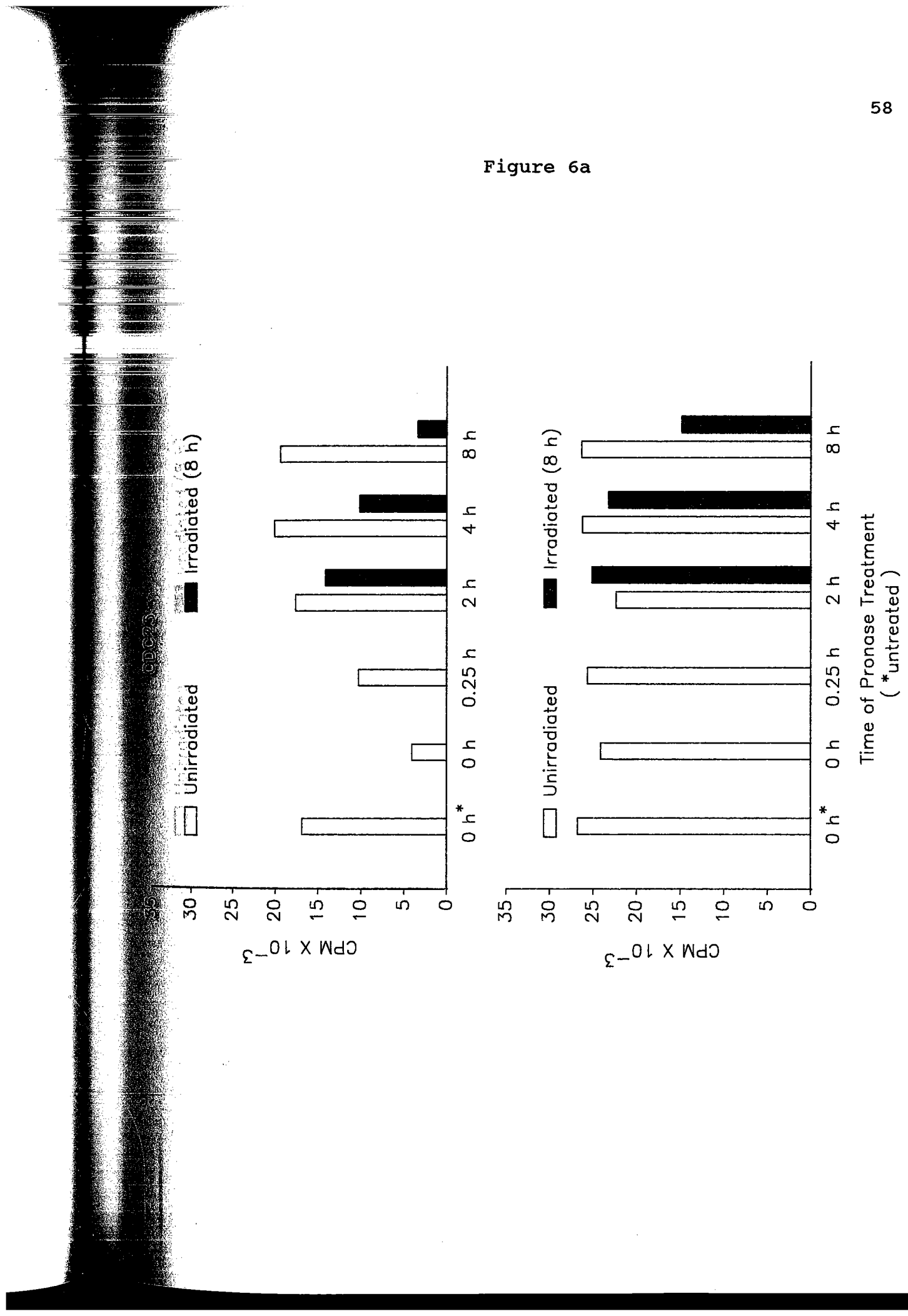
Unirradiated
Irradiated (8 h)
CPM X 10^-3
0 h*
0 h
0.25 h
2 h
4 h
8 h
Time of Pronase Treatment
(*untreated)

Figure 6b

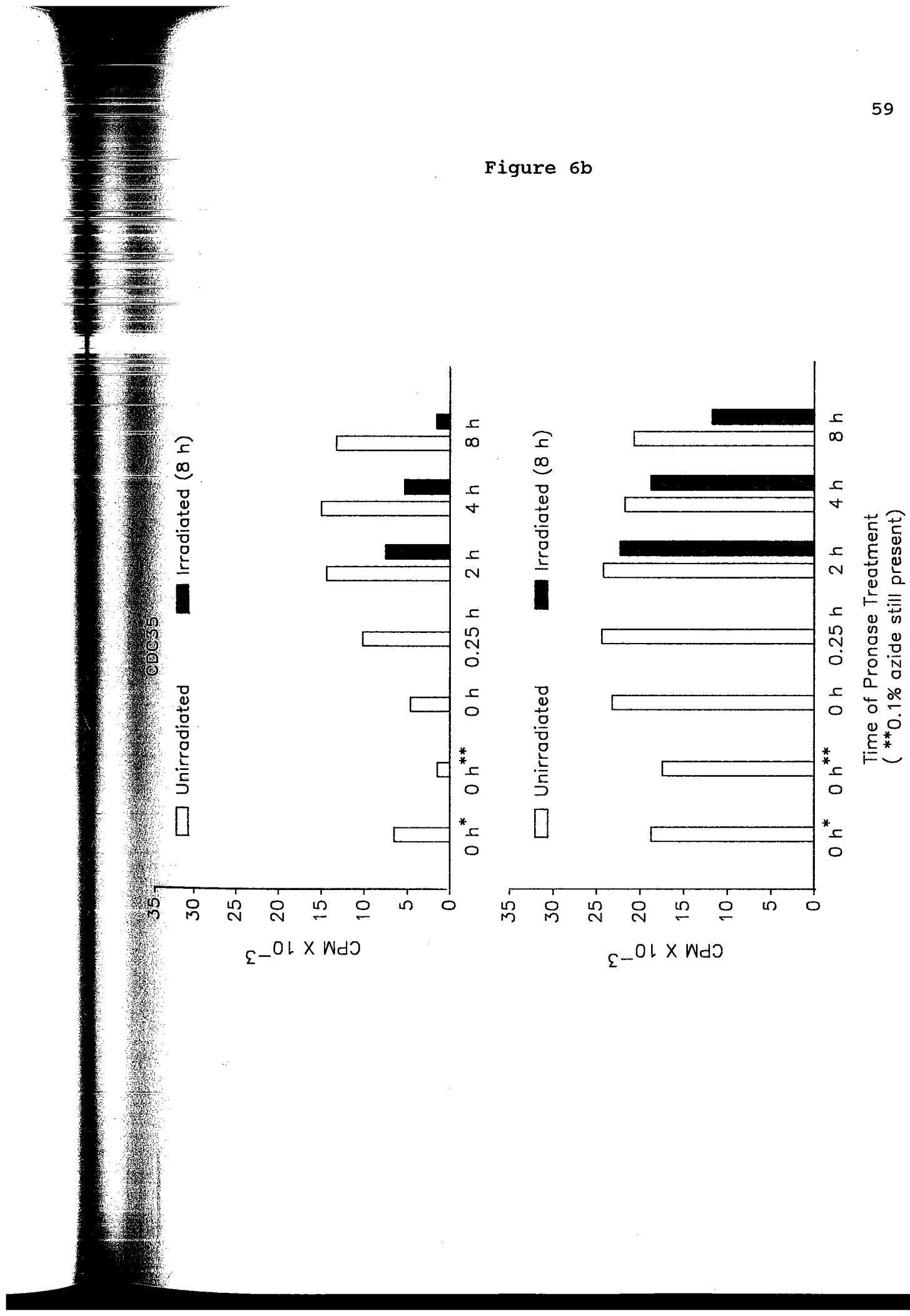
CDC35
Unirradiated
Irradiated (8 h)
CPM X 10^-3
0 h* 0 h** 0 h 0.25 h 2 h 4 h 8 h
Time of Pronase Treatment
(**0.1% azide still present)

Figure 6. Antigen internalization by small B cells detected by susceptibility to pronase. Resting B cells were pulsed, as in figure 5, with rabbit $F(ab')_2$ anti-Fab: Cells in figure 6a were pulsed with 0.01 µg/ml (top figure) or 0.1 µg/ml (bottom figure) of antigen. In figure 6b cells received 0.1 µg/ml (top figure) or 1.0 µg/ml (bottom figure) of antigen. While still at 4°C, the antigen was washed away. Either immediately, or at 0.25, 2, 4, or 8 hours of a 37°C incubation, the pulsed B cells were treated with pronase, and the 37°C incubation continued for the remainder of 8 hours. One half of each cell sample was irradiated with 2000 rad at the end of 8 hours to stop further processing. T cells, CDC25 (a) and CDC35 (b), were then added and lymphokine secretion monitored. In the absence of antigen, untreated B cells induced a background response of 1,247 counts (a) and 1,185 counts (b) which were not subtracted.

subtantially reduced at higher doses of antigen (Fig. 6). While pronase treatment and irradiation, both at 8 hours, eliminates the response of T cells to small cells pulsed with low doses of antigen, use of a ten fold higher dose of antigen restores the response to a level equivalent to or higher than that of small cells treated with pronase at two hours (Fig. 6). One possible explanation for this could be the failure, in some instances, of irradiation to prevent processing at the higher doses (Fig. 5a) combined with the failure of pronase to to totally remove antigen, also at these doses (Fig. 7a). The inhibitory effect of pronase at low doses of antigen does not appear to be due to an alteration of Ia on the B cell surface, or to the failure of irradiated B cells to replace altered Ia or some other surface molecule required for presentation. Pronase treatment, although it lowered background staining, did not alter the ability of an Ia-specific antibody to bind to Ia (Fig. 7b), or the ability of an allospecific T cell clone to respond to alloantigen on irradiated presenting cells (Fig. 7c).

Figure 7a

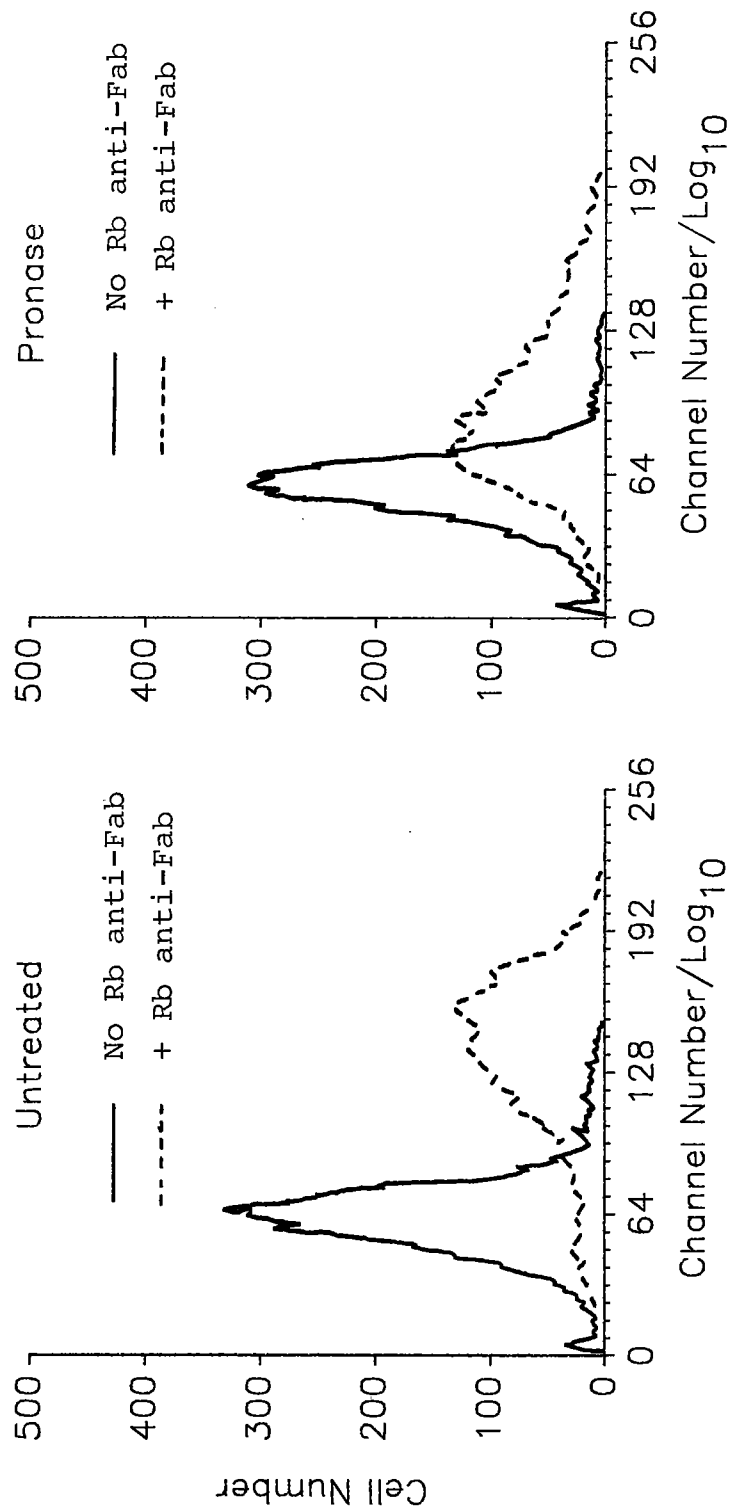
Untreated
Pronase
No Rb anti-Fab
+ Rb anti-Fab
Cell Number
Channel Number/Log10
0
64
128
192
256
100
200
300
400
500

Figure 7b

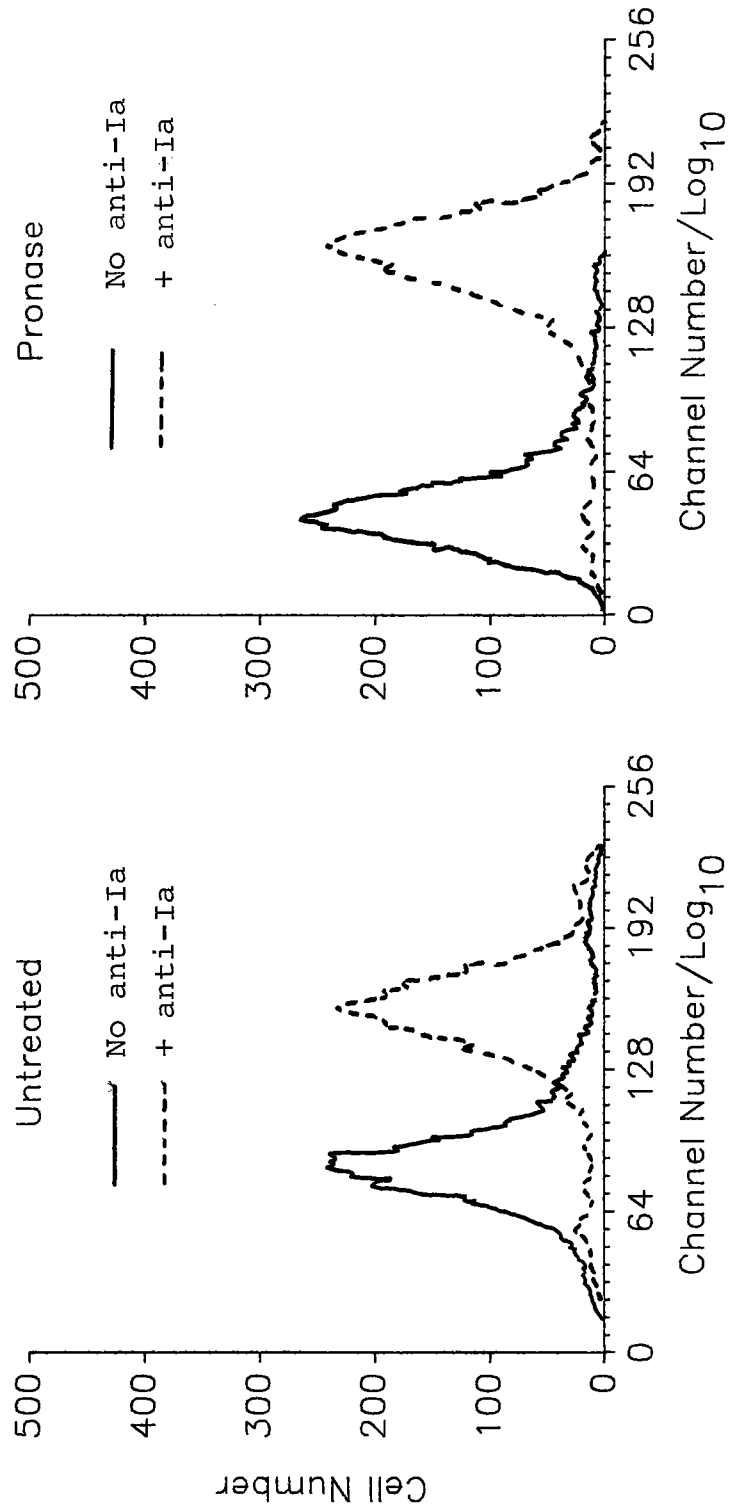
Untreated
Pronase
No anti-Ia
+ anti-Ia
Cell Number
Channel Number/Log10
0
64
128
192
256
100
200
300
400
500

Figure 7c

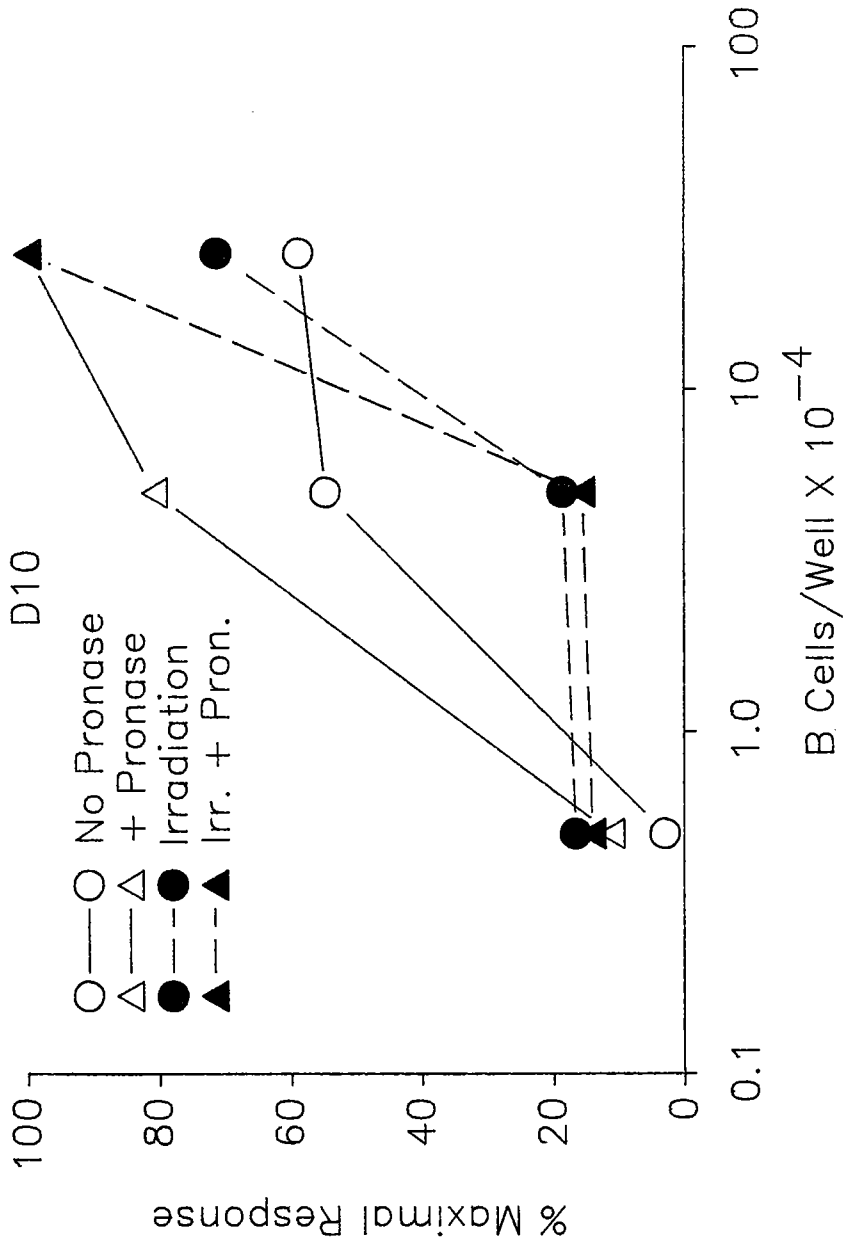
D10
No Pronase
+ Pronase
Irradiation
Irr. + Pron.
% Maximal Response
100
80
60
40
20
0
0.1
1.0
10
100
B Cells/Well X 10^{-4}

Figure 7. The effect of pronase treatment on Ia and antigen. Small B cells were divided in half, and one half was treated with pronase following a 30 minute, 4°C pulse with 1.0 µg/ml $F(ab')_2$ anti-Fab (a) or in the total absence of an antigen pulse (b and c). B cells were then stained for rabbit Ig (a) or Ia (b) using fluorescein conjugated goat anti-rabbit Ig or rat anti-Ia respectivily. The effect of pronase on the response of an alloreactive T cell line (D10) was also measured (c) by monitoring lymphokine secretion following the addition of T cells to plates containing pronase treated or untreated CBA/N X A.BY ($H\text{-}2^k$ X $H\text{-}2^b$) B cells.

PRESENTATION IS CHLOROQUINE SENSITIVE.

To determine if the antigen, once internalized, enters an acid compartment, chloroquine, a lysomotropic agent which raises lysosomal pH, was used to block processing. To do this experiment I used a method similar to that used by Chesnut et al. (155) in which it was shown that chloroquine did inhibit the presentation of processed antigen by B cell lymphomas. In this case, resting B cells were treated with chloroquine during a 4 hour antigen pulse. The antigen and chloroquine were then washed away and T cells were added. To be certain that inhibition was not owing to irreversible damage by chloroquine to the presenting B cell, antigen was added back to duplicate samples. At concentrations of 0.02mM to 0.07mM chloroquine, and doses of antigen which do not induce maximal responses, inhibition of presentation by chloroquine was observed (Fig. 8).

Figure 8

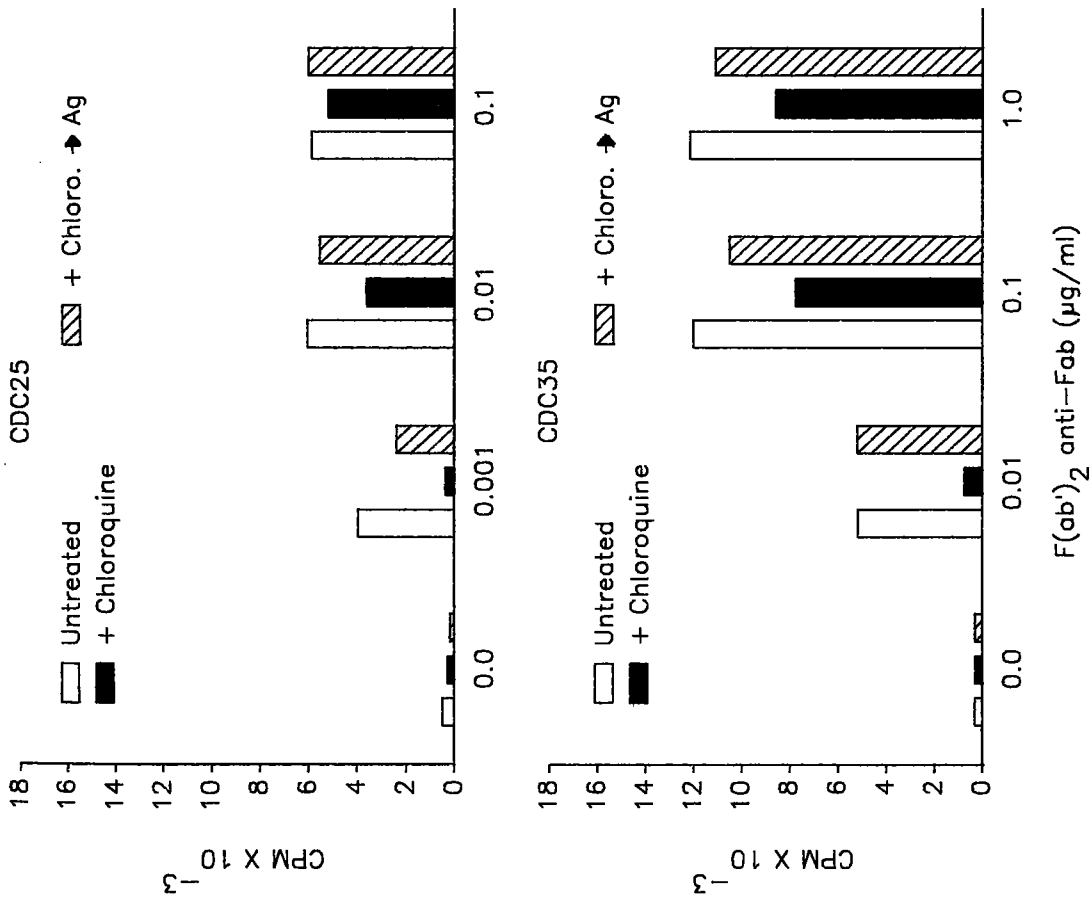
CDC25
Untreated
+ Chloroquine
+ Chloro. → Ag
CPM X 10⁻³
0
2
4
6
8
10
12
14
16
18
0.0
0.001
0.01
0.1
CDC35
Untreated
+ Chloroquine
+ Chloro. → Ag
CPM X 10⁻³
0.0
0.01
0.1
1.0
F(ab')2 anti-Fab (µg/ml)

Figure 8. Inhibition of presentation by chloroquine. Resting B cells (1 X 10^6 cells/ml) were incubated for 30 minutes at 37°C in the presence of 0.02 mM (upper figure) or 0.07 mM (lower figure) chloroquine (Sigma) in RPMI 1640. Rabbit anti-Fab was then added, and the incubation continued for 4 hours. Both antigen and chloroquine were then washed away and the cells resuspended in RPMI 1640. B and T cells were then added in duplicate to 96 well plates. To one set of plates, antigen was added back. Cultures were then incubated, and lymphokine secretion measured.

NATIVE ANTIGEN IS NOT PRESENTED.

To determine if native antigen is being presented, affinity purified goat antibody against native rabbit Ig was added to cultures containing pulsed B cells 30 minutes prior to the addition of T cells. If native rabbit Ig is being presented to the T cells, one would expect the goat anti rabbit Ig to bind and block presentation to the T cell (174). Up to a 3000 fold excess of the blocking antibody did not inhibit presentation (Fig. 9).

Since the presentation of rabbit Ig to our T cells is paticularly sensitive to fixation (Table 3), and previous studies using B cell lymphomas, fixation, and OVA as antigen have shown that the T cell lymphoma DO 54.8 can respond to digested, but not intact OVA on the fixed B cell, I decided to use this system to test whether our T cell lines respond to native rabbit Ig on the surface of the antigen presenting B cell lymphoma. Attempts to induce antigen presentation on fixed B cells using native rabbit Ig were not successful despite the fact the same fixed B cells did present a tryptic digest of OVA to the OVA-specific T cell hybrid DO 54.8. Our T cell line,

Figure 9

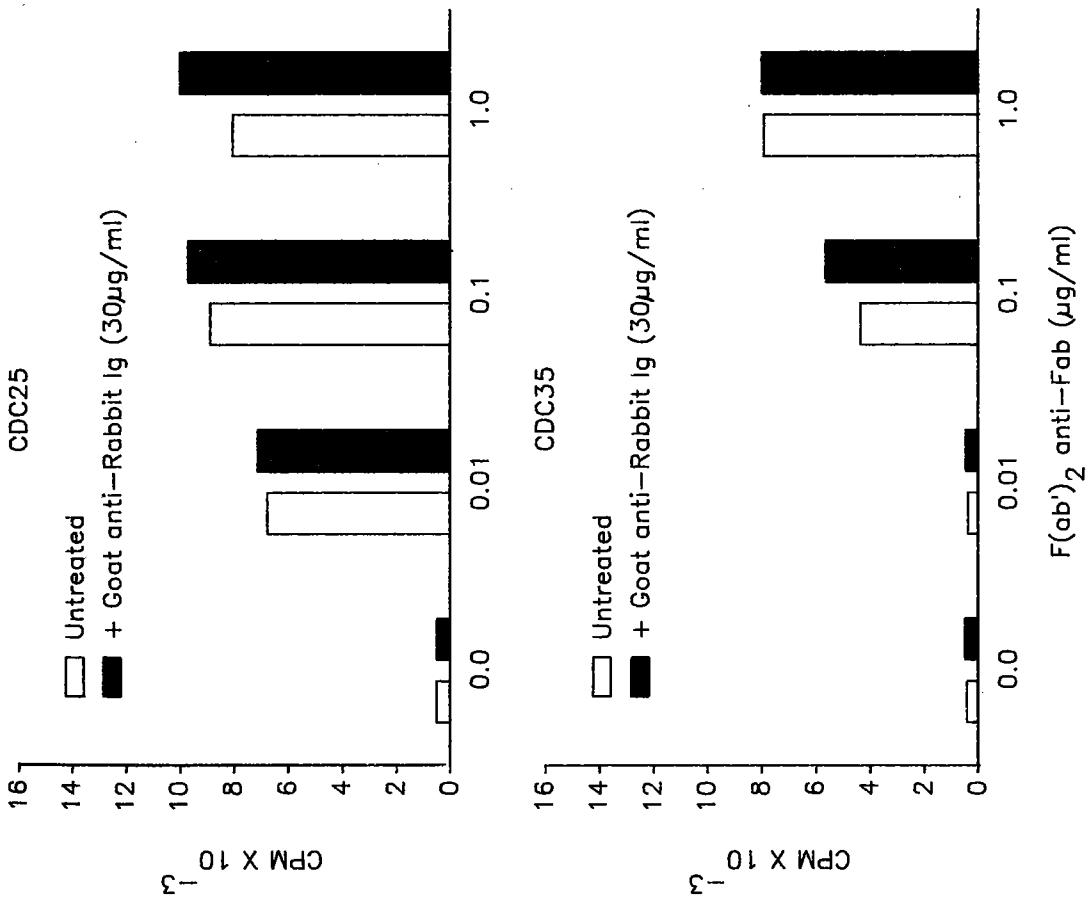
CDC25
Untreated
+ Goat anti-Rabbit Ig (30µg/ml)
CPM X 10⁻³
0
2
4
6
8
10
12
14
16
0.0
0.01
0.1
1.0
CDC35
Untreated
+ Goat anti-Rabbit Ig (30µg/ml)
CPM X 10⁻³
0
2
4
6
8
10
12
14
16
0.0
0.01
0.1
1.0
F(ab')2 anti-Fab (µg/ml)

Figure 9. The Failure of goat anti-rabbit Ig to block presentation of rabbit globulin. Resting B cells were pulsed for 30 minutes at 4°C with $F(ab')_2$ rabbit anti-Fab as in figure 5, and then incubated 8 hours at 37°C. Following this incubation, and 30 minutes prior to the addition of T cells, B cells were put into 96 well plates and 30ug/ml of $F(ab')_2$ goat anti-rabbit Ig were added to each well. Subsequently, T cells were added and lymphokine secretion measured.

CDC35, also failed to respond to the tryptic digest, but did respond to antigen on fixed B cells if fixation was preceded by an antigen pulse and 37°C incubation for 20 hours (Table 4).

Attempts to isolate a digest which could be recognized by our T cell lines on fixed B cells was not successful. However, it was possible, using heavy and light chains, and different allotypes of rabbit Ig, to determine that CDC35 recognizes a site within the light chain constant region of rabbit Ig. CDC35 failed to respond to purified heavy chain, but did respond to purified light chains. CDC25 appeared to respond to both (Fig. 10). This latter observation might also be due to contamination of light chains with heavy chain since CDC25 showed a strong preference for the latter. Both CDC25 and CDC35 detected changes in sequence within the heavy chain variable regions and the light chain constant regions respectively of rabbit Ig as indicated by their reduced responses to one of several rabbit Ig allotypes which differ between each other only in these regions (175,176) (Table 5).

TABLE 4

The effect of fixation on presentation of native antigen[a]

Fixative		0.05% Glut.		0.017% Glut.	
Treatment	T Cell	DO 54.8	CDC35	DO 54.8	CDC35
	Antigen	(OVA)	(NRG)	(OVA)	(NRG)
Unfixed	None	806	479		
	Native	39973	22402		
Postfixed	None	252	326	459	491
	Native	8395	12788	6777	15441
Prefixed	Native	468	685	767	508
	Digest[b]	3913	790	8952	663

[a] A20 B cell lymphomas were incubated for 20 hours at 37°C either in the presence (1.0 mg/ml) or absence of antigen. B cells were then fixed with either 0.05% or 0.017% glutaraldehyde as described in materials and methods.

[b] Tryptic digests of NRG and OVA were prepared as previously described (154).

Figure 10

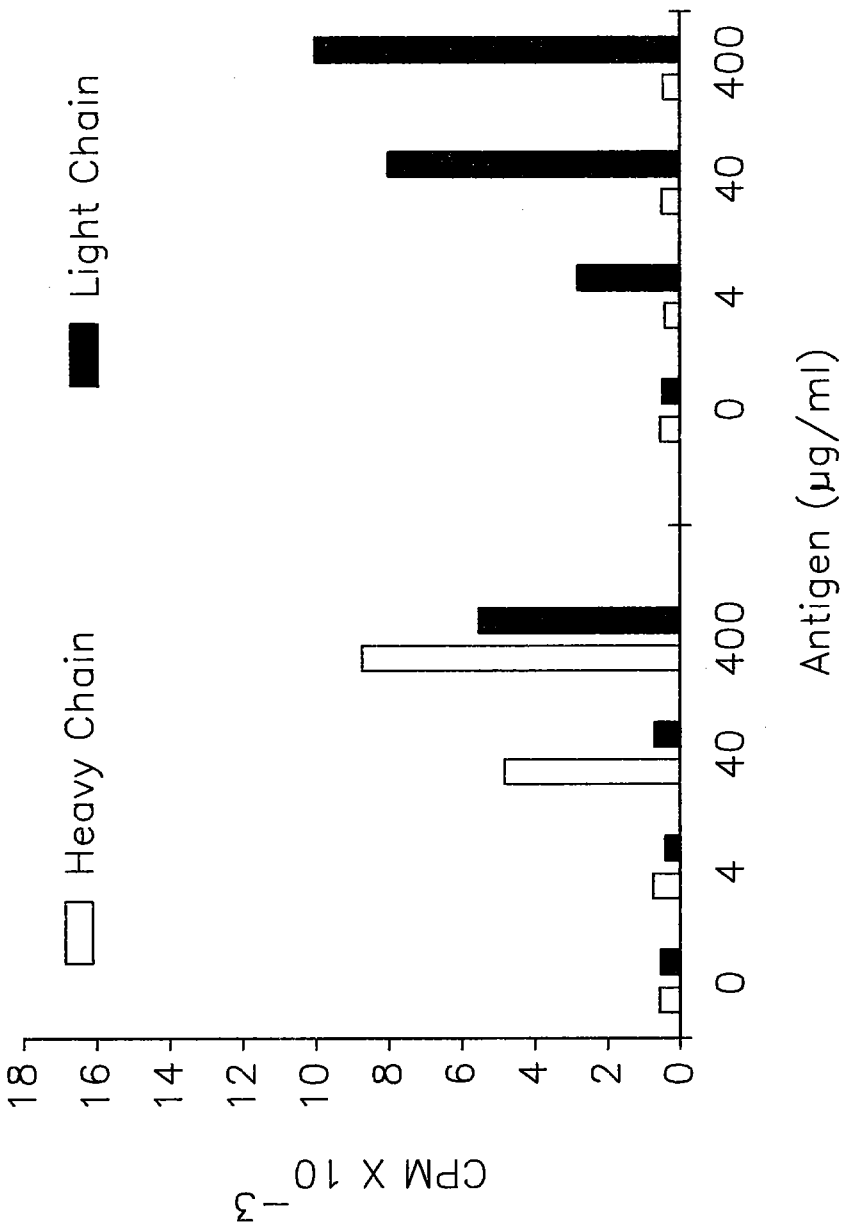
CDC25
CDC35
Heavy Chain
Light Chain
CPM X 10⁻³
0
2
4
6
8
10
12
14
16
18
0
4
40
400
0
4
40
400
Antigen (µg/ml)

Figure 10. The specificity of T cell lines for the heavy and light chains of rabbit Ig. B cells, T cells and heavy and light chains of rabbit Ig were combined in 96 well plates. The ability of our T cell lines to recognize these antigens was measured by assaying subsequent lymphokine secretion.

TABLE 5

The response of T cell lines to different rabbit Ig allotypes[a]

T Cell	Allotype	Antigen Dose (µg/ml) 500	250	125
CDC25	a1/1 b4/4	5321	3141	2185
	a2/2 b4/4	7714	3058	2309
	a3/3 b4/4	6427	1142	946
CDC35	a2/2 b4/4	5728	568	347
	a2/2 b5/5	4774	340	190
	a2/2 b6/6	3105	133	85
	a2/2 b9/9	568	113	94

[a] B and T cells were plated as indicated in materials and methods. The antigen was then added, and subsequent lymphokine secretion measured.

ANTIGEN IS NOT BOUND TO THE B CELL RECEPTOR AT THE TIME OF PRESENTATION.

To determine if antigen is bound to the B cell receptor at the time of presentation, the receptor was removed from the B cell surface with goat anti-Fab either before or after the antigen pulse. If the antigen is bound to membrane Ig at the time of presentation the latter treatment should remove it with the receptor, eliminating presentation (147,162). Addition of 30 µg/ml of goat anti-mouse Fab before the antigen pulse eliminated presentation of rabbit anti-IgM (Fig. 11). This was not the case if goat anti-Fab antibody was added after the pulse. The failure of goat anti-Fab to eliminate presentation after the pulse was not owing to steric interference by rabbit anti-IgM (the antigen), since flourescence staining did not demonstrate binding competition between these two antibodies (Fig. 12).

Figure 11

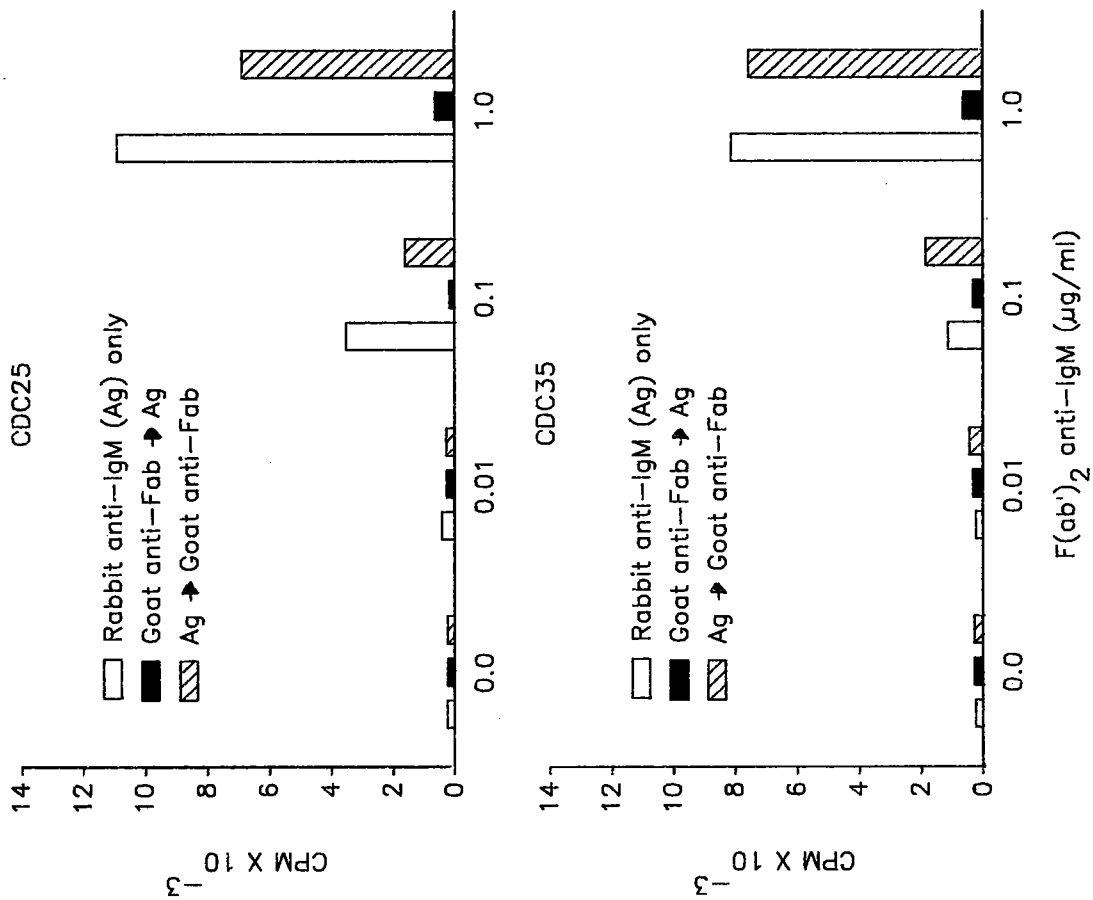
CDC25
CDC35
Rabbit anti-IgM (Ag) only
Goat anti-Fab → Ag
Ag → Goat anti-Fab
CPM X 10⁻³
0
2
4
6
8
10
12
14
0.0
0.01
0.1
1.0
F(ab')2 anti-IgM (µg/ml)

Figure 11. The failure of goat anti-mouse Fab to block the presentation of rabbit anti-mouse IgM following the antigen pulse. B cells were divided into two groups. One group of resting B cells was pre-incubated for 30 minutes at 37°C with $F(ab')_2$ goat anti-mouse Fab (30 µg/ml); the other group received no goat antibody. Subsequently, each group was pulsed with $F(ab')_2$ rabbit anti-IgM as described in figure 5. Following an 8 hour incubation at 37°C, the group which had not been preincubated with goat anti-Fab was further divided into two groups. One received goat anti-Fab, the other did not. Cells were then incubated an additional 30 minutes at 37°C, T cells were added, and levels of lymphokine secretion determined.

Figure 12

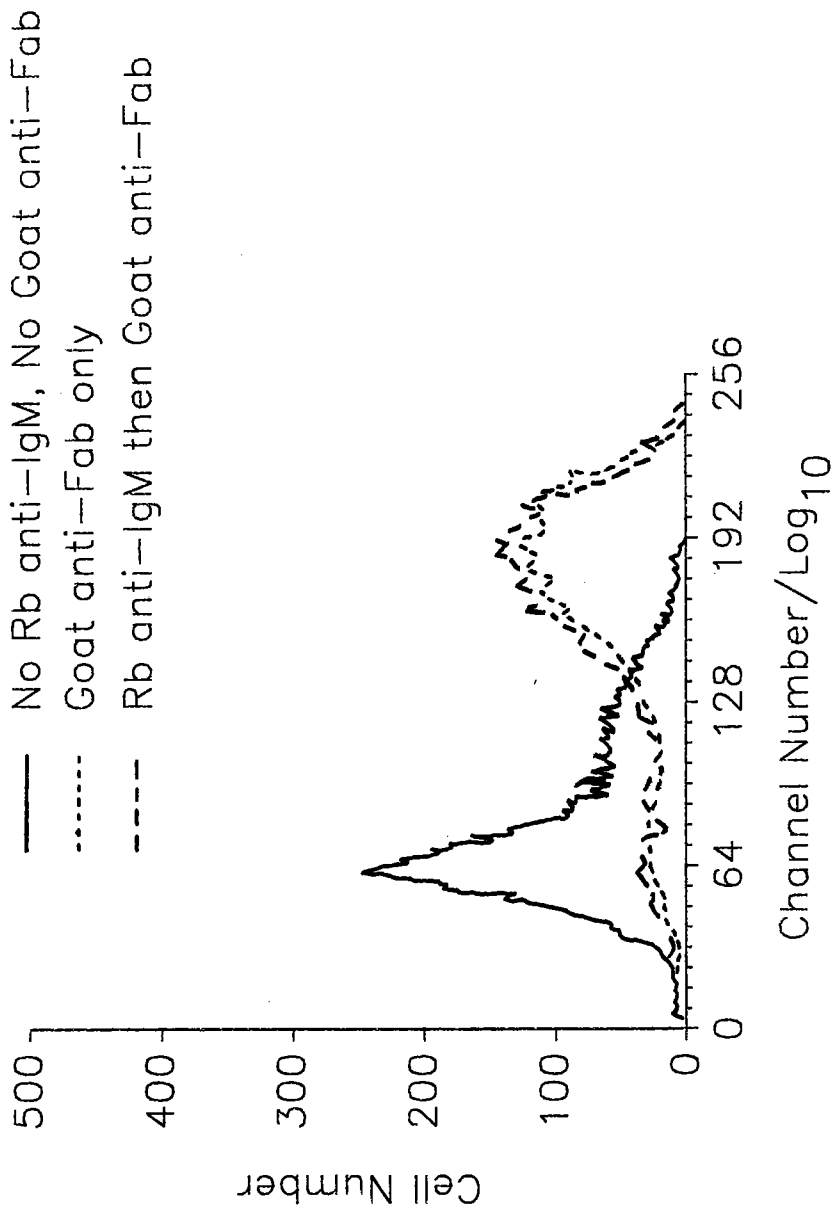
No Rb anti-IgM, No Goat anti-Fab
Goat anti-Fab only
Rb anti-IgM then Goat anti-Fab
Cell Number
500
400
300
200
100
0
0
64
128
192
256
Channel Number/Log10

Figure 12. The lack of binding competition between goat anti-mouse Fab and rabbit anti-mouse IgM. To B cells in RPMI 1640 plus 0.1% sodium azide, 1.0 µg/ml of $F(ab')_2$ rabbit anti-mouse IgM was added for 30 minutes at 4°C. The rabbit antibody was then washed away with HBSS plus 0.1% sodium azide, and the cells resuspended in RPMI 1640 (0.1% sodium azide). 30 µg/ml of goat anti-mouse Fab was then added for 30 minutes at 4°C, the cells were washed, and were then stained with fluorescein conjugated anti-goat Ig as described in materials and methods.

DISCUSSION

ANTIGEN PRESENTATION IN B CELL ACTIVATION

In order to produce antigen-specific antibody to a thymus-dependent antigen the B cell must first be able to function as an antigen-specific antigen presenting cell for the helper T lymphocyte. Evidence that this can occur has been provided by in vitro studies in which it has been shown that B cells from a number of sources, including macrophage-depleted spleen cells (127), primed lymph node B cells (161), Epstein Barr virus transformed B cells (147), normal antigen-specific B cells (162), and in our laboratory, size-selected small B cells (137,138), do present antigen very efficiently when it is initially bound to the B cell receptor. In addition, it has also been demonstrated that presentation by normal B cells can lead to a vigorous antibody response (137,138,163). At the outset of this study, however, it was unclear at what stage in B cell activation the B cell can present antigen in order to receive T cell help.

THE ANTIGEN PRESENTING B CELL

Normal B Cells

Normal B cells represent a diverse population of cells which vary in a number of respects including size and state of activation. Studies by Abbas et al. (162,163) using antigen-specific B cells isolated from normal B cell populations have clearly demonstrated that these cells do function very efficiently as antigen presenting cells, and that this presentation does lead to the production of antigen-specific antibody. In addition, these cells process antigen, as indicated by a number of criteria. First, normal antigen-specific B cells fixed or irradiated prior to the antigen pulse fail to present antigen. Second, antigen intially bound to membrane Ig is no longer bound to the B cell receptor at the time of presentation. This suggests that presentation by the normal antigen-specific B cell is sequential rather than simultaneous. Antigen binds to the B cell receptor, is processed, and then is presented to the T cell in the context of class II MHC molecules, but is no longer associated with the B cell receptor.

Resting B Cells

Recent studies have suggested that in order for the B cell to present antigen to the T cell, the B cell must first be activated (131,136,164,165). This implies that cognate interaction between the B cell and the T cell, and the delivery of T cell help, cannot occur with the majority population of small, resting B cells until those cells become activated by antigen, bystander effects, or some other signal (131).

In prior studies using rabbit Ig as antigen and rabbit Ig-specific T cell lines, it appeared that size-selected small B cells were capable of cognate interaction (137,138). To explain this, I considered the possibility that resting B cells are generally unable to process antigens, and that the rabbit Ig-specific T cells recognize native antigen bound to receptor Ig on the B cell surface. Processing of receptor bound antigen by the resting B cell had not been previously characterized in normal B cells which could be clearly defined as resting. While Abbas et al. (162) had shown that purified, hapten-specific B cells process and present hapten-carrier conjugates to carrier-specific T cells, these antigen-

specific B cells were obtained from the entire splenic B cell population, and so included large B cells that were activated in vivo. Also, these cells were isolated based on the ability of their receptors to bind antigen and could have been activated during the isolation procedure. Use of size-selected small B cells and rabbit anti-Ig as a polyclonal antigen analog circumvents this problem, and allowed me to show that small resting B cells do process antigen.

Processing by small B cells appears to be very similar to that of other antigen presenting cells in that antigen must bind to the cell, be internalized into an acid compartment, and be returned to the cell surface in a modified form, which can then be recognized by the T cell in the context of MHC class II molecules.

Using the observation that antigen processing requires an intitial period of incubation at 37^{o}C, and that, in B cells, processing is more radiosensitive than presentation of processed antigen (135,162), I have shown that receptor mediated antigen processing in small B cells takes between 2 and 4 hours after an antigen pulse, with presentation reaching a maximum by 4 to 8 hours (Fig. 5).

Antigen processing appears to be the rate-limiting step in antigen presentation. Previously, it was observed

that divalent $F(ab')_2$ fragments of anti-Ig were presented at the same rate as monovalent Fab' fragments, as measured by the onset of lymphokine secretion at 10 to 12 hours (137), even though the divalent form of anti-Ig is capped and endocytosed in a few minutes while much of the monovalent antibody persists on the cell surface for at least an hour (177). In these studies I observed no difference in the rate of antigen processing between divalent and monovalent antigen measured at 4 hours (Fig. 5c). The rapid endocytosis of capped antigen does not accelerate processing under the conditions we used, and monovalent antigen appears to enter at a rate that saturates the processing pathway. However, the observation that $F(ab')_2$ anti-IgD is presented more rapidly to CDC25 than $F(ab')_2$ anti-IgM (Fig. 5a) and the fact that there are higher levels of IgD versus IgM on the B cell surface (160) could indicate that antigen dose is rate limiting. Alternatively, antigen bound to surface IgD may follow a different pathway than antigen bound to IgM, and/or there may be greater difficulty in processing anti-IgM (ie: there is a need for multiple passages through the processing pathway). In fact CDC35, which responds to a different determinant on rabbit globulin than CDC25 (Fig. 10) did not detect any differences in

processing time between antigen bound to IgD and IgM (Fig. 5b). This implies that the determinant CDC35 sees is more easily processed than the determinant seen by CDC25 when bound to surface IgM. The consistent reduction of presentation by 12 to 20 hours of incubation (Fig. 5) suggests that once processed antigen reaches the surface, it remains for as little as 4 hours before being lost to further processing or the surrounding medium. This conclusion is in basic agreement with observations by Buus et al. (101) that the half-life of antigen bound to Ia at 37^{o}C is between 5 and 10 hours.

The observation that presentation could still occur at higher doses of antigen despite irradiation at time 0 (immediately after antigen binding) (Fig. 5a) could have a number of explanations. It might be that in the presence of large amounts of antigen enough antigen is still processed by small cells to still allow presentation. Alternatively there may be a small number of radioresistant large cells which require the higher doses of antigen in order to present. I cannot exclude the latter possibility. However, based on the radiosensitivity of presentation by my small B cells at the lower antigen doses, and the observation that anti-Ig does not induce radioresistance by 8 hours in these cells

(Table 3), it seems clear that at the lower antigen doses I am looking at processing and presentation by resting B cells.

In addition to the requirement of 2-8 hours for processing, internalization also appears to be part of the processing pathway. This is based on the observation that antigen pulsed cells are able to present antigen following pronase treatment at 2, 4, or 8 hours of incubation, but not if treated with pronase immediately after the pulse when all the antigen would still be on the cell surface (Fig. 6). However, if processed antigen is resistant to pronase, then processing could be occuring on the cell surface. To test this idea, I divided treatment groups in two and irradiated one set of samples at 8 hours to stop further processing. This eliminated three fourths of the T cell response to presenting B cells which had been treated with pronase just before irradiation (Fig. 6). This result implies that processed antigen is sensitive to pronase, and that reexpression of internalized antigen, which occurs over an extended period of time, is sensitive to irradiation. Alternatively, small B cells treated with pronase and irradiated at 8 hours do present antigen when the antigen dose is increased (Fig. 6a). There are a number of possible reasons for this. Pronase fails to

totally remove the antigen at higher doses (Fig. 7a), and irradiation does not totally inhibit presentation, also at these doses (Fig. 6a). Thus, presentation, in this case, may be due to antigen which is still processed and presented despite these treatments. The observation that B cells irradiated and treated with pronase at 8 hours still can present rabbit Ig also suggests that the machinery necessary for presentation (other than antigen) is still intact. As has been shown previously (155), fluorescence staining of pronase treated cells with anti-Ia antibody indicates that pronase does not effect the level of class II expression on the B cell surface (Fig. 7b). This is consistent with the idea that the effect of pronase treatment is not due to removal of class II molecules. Pronase may remove some other important molecule from the B cell surface whose reexpression is radiosensitive. However, we found that irradiated, pronase-treated small B cells were just as effective as irradiated, untreated cells in stimulating an alloreactive T cell line (Fig. 7c).

Further support for processing comes from a number of additional observations: inhibition of presentation by chloroquine, the failure of our T cells to recognize native antigen, and the observation that the antigen is

not bound to the B cell receptor at the time of presentation.

Chloroquine is a weak base which, in its unprotonated form can penetrate the membranes of acidic compartments. Once inside these compartments, chloroquine becomes protonated. In this form it can no longer pass through membranes and is trapped (178). The buildup of chloroquine inside acidic compartments increases the pH (179) and presumably alters the activity of pH-dependent enzymes which may be involved in processing. Chloroquine also has a number of other effects on cells including: changes in ultrastructural morphology of intracellular vesicles (178), inhibiton of endocytosis (180, 181), inhibition of receptor recycling (182,183), and inhibition of class II processing (ie: Ii no longer dissociates from class II) (184). However, all of these effects may still be the indirect result of raising lysosomal pH. Thus, despite the additional effects of chloroquine treatment, the inhibition of presentation I have observed does add further support to the contention that rabbit Ig is internalized prior to presentation. It also implies that acidic compartments are important to processing of antigen by the resting B cell. In addition, antigen added subsequent to chloroquine treatment is presented,

indicating that inhibition is reversible and requires the presence of chloroquine during the antigen pulse (Fig. 8).

Our T cells do not appear to recognize native antigen. They do not respond to native NRG on fixed B cell lymphomas (Table 4), or to rabbit anti-Ig on small B cells fixed immediately after the addition of antigen (Table 2). If our T cells did see native antigen on the B cell surface, then antibody against native rabbit Ig should block presentation (174). Such attempts to block presentation were unsuccessful (Fig. 9). Finally, antigen is not attached to the B cell receptor at the time of presentation. Anti-mouse Ig, which removes sufficient amounts of the B cell receptor to prevent presentation when added before the antigen pulse, cannot block presentation of an equivalent amount of antigen when added after the pulse (Fig. 11).

A number of questions still remain regarding processing by these and other antigen presenting cells. Studies of processing as it applies to intracellular trafficking of Ia and antigen have not been done. Specifically, we still are not certain that antigen meets Ia inside the presenting cell. Also uncertain is whether antigen is presented on recycled or newly synthesized Ia. Unanue et al. (177,185) did carry out ultrastructural

studies of antigen internalization in the early 1970's, but this was prior to the discovery of MHC restriction. Cresswell has provided some indirect evidence that Ia and antigen meet inside the cell (186) by showing that neuriminidase, when attached to transferrin, cleaves sialic acids on both Ia and Ii following internalization. In regards to the question of whether antigen binds to recycled or newly synthesized Ia, Pernis has suggested that Ia and antigen are internalized simultaneously after antigen binding and subsequently meet inside the presenting B cell (187). However, this evidence is still considered preliminary by most researchers and confirming evidence has not been forthcoming.

Using ultrastructural methods (ie: colloidal gold and electron microscopy), I plan to address these questions in a more direct manner by following antigen and Ia into the presenting B cell and labeling both simultaneously in the same cell to determine where they go and if they enter the same organelles.

The failure by others to demonstrate presentation by the resting B cell may have a number of possible explanations. By "small resting B cells", we mean the majority population of small B lymphocytes in the spleens of our mice. To avoid contamination with larger

lymphocytes and other cell types, I typically take only 10 to 20% of the total number of T-depleted spleen cells. Since our small B cells have both the physical and functional characteristics of resting cells (7,138,160,167,188), and Fig. 4, I was not interested in a minority population of very small or very dense B cells which may be deficient in antigen presentation. Despite the fact our cells are resting at the time of isolation, Hans-Peter Tony in our laboratory considered the possibility that the presentation we see may be owing to the presence of activated B cells, either due to contamination with small numbers of large cells, or to activation by antigen or nonspecific signals delivered by the T cells or present in the medium. Since our T cell lines respond as well on a per cell basis to antigen presented by small as by large spleen cells (189) contamination by large cells cannot account for the activity of our small cell population. Activating signals delivered by antigen, or by the T cells or culture conditions prior to cognate interaction were also shown not to be necessary, since preincubation of small B cells with T cells but without antigen or in medium alone does not alter the kinetics of lymphokine production once antigen and T cells are present together (189).

Small cells may present some antigens but not others. Rabbit anti-Ig binds directly to the B cell receptor and is presented very efficiently (127,137,138). In the case of antigens which bind nonspecifically to the B cell surface, the ability of individual antigens to bind to the surface of the resting B cell may vary depending on their structure. Some antigens may bind poorly or not at all even at high concentrations (190). Alternatively, T cells may be heterogeneous in their ability to see antigen on resting B cells because of a need for an accessory cell function not provided by resting B cells (135) or because of fine specificity differences in their antigen receptors which may detect differences in class II molecules or processed antigen between resting and activated B cells (136,191).

Despite the failure by others to demonstrate presentation by the resting B cell, it is clear from these studies that the resting B cell is capable of processing and presenting antigen to a helper T lymphocyte without prior activation. This is consistent with the suggestion by Kishimoto (192) that the T cell secreted lymphokine IL-4 acts primarily on the resting B cell, and implies that the interaction between the resting B cell and the helper T lymphocyte may provide tbe initial signal in B cell activation.

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